FINAL REPORT

Cooperative Agreement No. 12-14-5001-272

Investigations on Leafhopper Vectors, Diagnosis, and Etiology of Western X-disease (buckskin) of Cherry Trees in California

PART ONE

(April 1, 1977 - January 31, 1979)

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PART TWO

(February 1, 1979 - March 31, 1981)

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The report summarizes the results of research conducted under Cooperative Agreement no. 12-14-5001-272, "Investigations on Leafhopper Vectors, Diagnosis, and Etiology of Western X-disease (Buckskin) of Cherry Trees in California".

The research accomplished during this project as reported in PART ONE was divided into two major areas: (1) the leafhopper vectors of the causal agent of X-disease and (2) the causal agent - presumably a mycoplasma or spiroplasma - of X-disease. The principal investigator of the first of these major topics was Dr. Alex H. Purcell, Entomologist, University of California, Berkeley. Participants in various phases of this research were Mr. Allan Finlay, Mr. Joseph Elkinton, Mr. Stephen Misari, Mr. Richard Kawin, Mr. Rollin Coville, and Dr. Charles Wu.

The study of the causal agent of X-disease proceeded under the supervision of Dr. Sherman V. Thomson, Plant Pathologist, Dept. of Plant Pathology, Berkeley. Mr. Doug Garret, Dr. Michael J. Davis, and Ms. Eileen Rabb completed various phases of the research reported here. Dr. George Nyland, Dr. B. C. Raju, and Mr. S. K. Lowe, Dept. of Plant Pathology, University of California, Davis, helped in many phases of this work. Mr. Wallace P. Schreader, Farm Advisor, San Joaquin County, and Dr. S. M. Mircetich, U.S.D.A., Dept. of Plant Pathology, University of California, Davis, were also very helpful, as were many others acknowledged later in this report. We are also grateful for unacknowledged assistance from many sources.

Principal investigators who conducted the research reported in PART TWO acknowledge the valuable assistance of Dr. B. C. Raju and S. K. Lowe at Davis; D. G. Garrott and Karen Gonot Suslow at Berkeley; and Wallace P. Schreader, Farm Advisor, San Joaquin Co., California.

BACKGROUND

Western X-disease (Cherry Buckskin) (XD) has been recognized as an often serious problem in California and in other parts of the United States since the 1930's. The disease was a major factor in the loss of commercial cherry production in Napa, Sonoma, and Solano counties and continues to be a serious problem in California's major cherry producing area in the San Joaquin Valley centered near Stockton.

The pattern of occurrence of X-disease suggested from the beginning that insects were responsible for the spread of X-disease. In the early 1950's, a number of leafhopper species were identified as vectors of the X-disease agent (XDA). This list has continued to lengthen. The principal insect species responsible for the spread of X-disease in California has never been determined.

Additional herbaceous plant species have also proven susceptible to infection with the XDA. In the eastern U.S., wild chokecherries seem to provide an important reservoir for the spread of X-disease into stone fruit orchards. In California, such a reservoir has not been identified, if one in fact is present.

Until the late 1960's the causal agent of X-disease was presumed to be a virus. Antibiotic therapy of diseased trees and electron microscopy of infected tissues suggested, however, that a mycoplasma probably caused the disease.

Repeated attempts to isolate the causal agent and grow it in pure culture have not been successful. Such an accomplishment possibly would make diagnosis of X-disease easier and more reliable. Most importantly, the isolation and culture of the causal agent would open new avenues for investigation of X-disease.

The general objectives of this project were to identify the principal vectors responsible for the spread of X-disease and to learn something of their biology, to develop methods to diagnose X-disease in cherry and to culture the causal agent (XDA) on artificial media. The specific objectives of the project (underlined), the efforts made to accomplish them, and the results of these efforts, are described in the remainder of this report.

A. Leafhopper Vectors

1. Sampling procedures for leafhopper populations and movements.

Five types of sampling methods were evaluated for their usefulness in estimating leafhopper abundance and movements in cherry orchards: sweep net, suction samples, visual search, knockdown with an aerosol mist of quick-acting insecticide, and sticky traps. The first four methods provided estimates of abundance in specific habitats such as orchard cover

vegetation or cherry foliage. Trapping was a measurement of net abundance and flight activity.

2. Leafhoppers most frequently found in or near cherry orchards in San Joaquin County.

The leafhoppers collected in all sampling methods were identified and compiled in a list.

METHODS

Sticky Traps

From a questionnaire survey by mail in 1977 of San Joaquin County cherry growers, we selected nine orchards that normally did not use insecticides. Five of the orchards were cultivated for weed control; in the remaining four orchards, surface vegetation was periodically mowed during the growing season for weed control. Two blocks, each consisting of three adjacent rows of 30 trees, were located in each orchard: One block was centered 5 rows from one edge of the orchard and the other 15 rows from the same edge.

Yellow sticky board traps were hung approximately 1.8 m high in a random quadrant in each of four randomly selected trees per block. The traps were 12.7 x 25.4 x .3 cm plywood, painted with "Bright Yellow" enamel (Fuller-O'Brien Paint, San Francisco, CA) and coated on both sides with "Stickem Special®" (Michel & Pelton Co., Emeryville, CA). The traps were checked weekly or biweekly and all leafhoppers removed and identified. Traps were changed as needed, usually biweekly. In one experiment, seven yellow sticky traps were wired together end to end and suspended from a tree with the lowest trap just above ground level. A strip of 0.6 cm mesh wire the same width and length as the seven-board sticky trap was coated with Stikem and humg just above ground level. Two sets each of long yellow and long mesh traps were placed in one mowed and one disked orchard.

Samples of Orchard Cover -- One cultivated orchard and one "sod culture" (mowed) orchard were selected for comparisons of leafhopper populations present on orchard cover vegetation. Two methods of collecting surface samples were (1) a 38 cm diameter insect sweep net and (2) a gasoline hand-held D-vac® suction collector. A single sweep net sample was fifty 180° sweeps between sticky trap locations. A D-vac® sample consisted of twenty-five placements of the D-vac® collector flush to the ground. Four sweep net and D-vac samples were taken in each of two blocks in the two orchards at the same date that sticky traps were checked in all orchards. Occasionally, sweep net and D-vac samples were omitted from the disked orchard immediately after cultivation because of lack of vegetation. The insects were transported to the lab in plastic bags, killed in dry heat, sorted and all adult leafhoppers identified and counted. Initial leafhopper identifications made by Ray Gill,

California Dept. of Agriculture, were used to establish a reference collection for further identifications. Mr. Gill also made determinations of specimens we were unable to identify. Specimens of the genera Empoasca, Dikrella, Edwardsiana, and Balclutha were initially misidentified from sticky trap collections. These specimens were counted or tabulated in a single group since the earlier identifications could not be repeated.

Direct Samples of Cherry Canopy -- A backpack mistblower model 250A (Curtis Dyna-products Corp, Westfield, Indiana) loaded with insecticide was used to knock down leafhoppers present in the cherry canopy onto a canvas ground cloth. A 6 m square canvas cloth was placed under the tree. One liter of 1.5% pyrethrin (28.5 ml of Pyrenone®, FMC Corp., Middletown,NY) in 1.0 l of water was blown in an aerosol mist over the tree. The tree was then immediately shaken and the ground cloth quickly searched for leafhoppers that had fallen onto the ground cloth.

Fifty sweeps of cherry foliage concentrated on a single tree where possible was another direct sampling method for leafhoppers in the cherry canopy.

Orchard Variables -- At each sampling date, the state of recent orchard cultivation or mowing and irrigation were noted, as well as the dominant weed species and their growth. The proximity and direction of adjacent crops were also recorded for each orchard.

RESULTS

Comparisons of Sampling Methods -- Both sweep net and D-vac samples of the orchard surface vegetation yielded similar compositions of leafhoppers, but differed markedly compared to the proportions of leafhoppers species collected in sticky traps. This is demonstrated for five representative species in Fig. 1. Table 1 lists the leafhopper species collected by sweep net, D-vac, and sticky trap in the two intensively sampled orchards. Total catch rather than average per sample figures are listed in Table 1 because no D-vac or sweep net samples were taken immediately after disking. Amblysellus grex (Oman) was the most common species in D-vac and sweep net samples, but much less prevalent on sticky traps. The second most abundant species was Deltocephalus sonorus (Ball), which also was rare in sticky trap samples. On the other hand, the grape leafhopper, Erythroneura elegantula Osborn, made up a higher percentage of the total catch from sticky traps than from sweep net or D-vac samples.

The great disparity between catches of sticky traps hung from cherry trees and sweep net or D-vac samples of the vegetation beneath these trees suggested that the leafhoppers most abundant on surface vegetation seldom ventured into the higher foliage of cherry trees. Traps that were knocked to the ground by wind, however, often captured enormous numbers of leafhoppers. To estimate the effect of trap height, we hung 1.8 m long yellow and 0.6 cm long mesh traps that extended to the ground below. The number of leafhopper species captured at various heights are shown in Fig. 2. Relative diversity of leafhoppers to D-vac and sweep net samples were similar. At greater heights, however, C. montanus, the grape leafhopper, E. eleagantula, and several species of very small leafhoppers, chiefly Empoasca and Dikrella spp. were most prominent.

The catches on the sticky mesh trap (Fig. 2B) followed the same trends with respect to height noticed in yellow traps but catches were lower for all species compared to catches on yellow traps (Fig. 2A) (Wilcoxan matched pairs signed ranks test; $P < .01$). As previous studies (Southwood, 1966; Alverson et al., 1977) have indicated, yellow appears to be strongly attractive to most leafhopper species, although there is not sufficient data for many species collected in this study to answer this point.

In the 2 orchards in which three sampling methods were evaluated concurrently, sticky traps detected the greatest number of species (Tables 1, 2). With the exception of *Aceratagallia* species, which were not collected by D-vac, all relatively common species were detected by each method. An extremely low average number (0.1/sample, n = 96) of *Exitianus exitiosus* (Uhler) were collected by D-vac compared to the average of 3.0/sample (n = 268) collected by sweeping. We noted that some large-bodied leafhopper species were able to crawl or leap from the collecting bag of the D-vac sampler while the machine was operating. As a rule, smaller insects rarely escaped.

The differences between the ratio of sweep: D-vac catches in mowed vs. disked locations (Table 1) was probably due to the more selective placement of D-vac samples in the often patchy or sparse vegetation of the disked orchard.

The comparison of sticky trap catches between clean-cultivated and permanent cover crop orchards (Table 2) revealed few major differences in catch due to weed control practices. Differences in sweep net and D-vac samples were more pronounced (Table 1) but could well be due largely to locality differences rather than the effects of weed cover. The short-term and longer-term influence of cultural practices and adjacent crops on leafhopper abundance and activity in cherry orchards will be discussed in detail in a later section.

X-vector species -- Of the leafhopper species known to be vectors of the X-disease agent, the two most commonly collected were *Colladonus montanus* (Van Duzee) and *Euscelidius variegatus* (Kirshbaum). *Colladonus montanus* was the most abundant and widespread of known X-vector species, particularly in sticky trap collections (Table 1). *Fieberiella florii* (Stål) was collected in low numbers only on sticky traps in two orchards. Only 16 *Colladonus geminatus* (Van Duzee) were collected from 5 orchards. The remaining known X-vector species: *Scaphytopius* spp, *Paraphlepsius* spp., and *Osbornellus borealis* De Long & Mohr were rarely collected. *Acinopterus angulatus* Lawson, recently shown to be an X-vector (Purcell, in press), was also rare, being collected chiefly from trefoil (*Lotus* sp.).

Direct Sampling of Cherry Foliage -- Until September, we uniformly failed to capture any leafhoppers by this method except for *Empoasca* and *Dikrella* species, which were present in very low numbers as adults. On Oct 18, 1977, we encountered low but regular numbers of *C. montanus* from cherry foliage. A total of 12 *C. montanus* were collected from 25 trees (25 sweeps/tree). The only other species collected were 3 *A. grex* and 5 *Empoasca* spp. At the same time, trap catches of *C. montanus* also increased sharply (Fig. 3).

On the same day that we detected relatively high numbers of C. montanus in cherry foliage we used the pyrethrin knockdown method to attempt to estimate per tree numbers of leafhoppers. These attempts were unsuccessful because few leafhoppers were knocked down by the insecticide mist. Furthermore, it was impossible to keep track of leafhoppers that moved onto the ground cloth from outlying surface vegetation along all edges of the cloth. We noted that this treatment induced an increased shed of leaves (~ 10%), during the following week. Similar attempts to use the pyrethrin knockdown method on other occasions were also unsuccessful.

Effects of cultivation on leafhopper diversity and abundance -- The rationale for sampling a number of clean cultivated and sod-culture orchards was to examine differences in the leafhopper populations on each of these different types of orchard vegetation. Surprisingly, there was little difference between weed control approaches in either the species of leafhoppers observed or their relative abundance in cherry trees. Figure 4 demonstrates that if anything, most leafhopper species were more abundant in clean cultivated orchard sticky trap catches than in grass cover orchards. The comparison of sweep and D-vac samples has already been mentioned (Table 1).

DISCUSSION

The sharp differences in the relative numbers of each leafhopper species detected by different sampling methods emphasize that the choice of sampling method should be determined not only by the leafhopper species of interest, but the intended purpose of the sampling regime. Our results indicate, for example, that either sweep net or D-vac samples might be suitable for estimating the relative abundance of leafhoppers present on vegetation near the ground but do not reflect accurately the relative abundance of leafhoppers in a different strata, such as in tree canopy. Direct sampling methods (sweep net, D-vac) are instantaneous estimates of the abundance of a given species at the time and place that the sample is taken. In contrast, sticky traps continuously estimate the cumulative abundance and activity of the insects trapped. The effects of extraneous factors such as time of day, temperature, wetness, and so on on sampling results have been well documented for the sweep net method. In addition to many of the same defects, sticky trap catches must be interpreted with the additional recognition that they are greatly influenced by behavior - specifically flight behavior. An illustration of this selectivity is that sticky traps capture only adult leafhoppers, since nymphs are flightless. Less obvious but no less important is that non-flying adults are not caught. Sticky trap catches reflect both abundance and activity. Estimates of abundance alone are better suited to direct sampling methods such as the sweep net.

The differential attractiveness of yellow traps to various leafhopper or other insect species presents problems in interpreting the significance of trap catches. Draeculacephala minerva was collected in insufficient numbers on sticky traps to estimate its relative preference for yellow traps

compared to mesh traps. Previous studies of this species in localities where it is very abundant (Purcell, unpublished data) indicate that it is not very sensitive to yellow and is captured in much higher numbers near the ground. The crepuscular flight behavior of *D. minerva* (Frazier, 1949) may explain this relative insensitivity to color, or at least to yellow. A similar situation may also explain somewhat the low numbers of *Euscelidius variegatus*, a known vector of the X-disease agent (Jensen, 1969), captured in yellow sticky traps. Previous yellow trap results in central California yielded similar results (Rice and Jones, 1972). One of us (Purcell, unpublished data) collected large numbers of *E. variegatus* attracted to lights in an abandoned prune orchard near Columbia, CA in August, 1977, which suggests significant nocturnal activity. Yellow sticky traps presumably would have a low capture rate for nocturnal insects.

The X-disease agent in California is most likely to be transmitted to cherry by adult leafhoppers because no leafhoppers in California are known to breed on cherry, and the latent period, or the time required between acquisition and inoculation (Jensen, 1953; 1969), is such that inoculation by nymphs would be rare. Vector flight activity, therefore, is presumably an important parameter in the spread of X-disease. Sticky traps are better suited for estimates of the product of abundance and activity than are more direct collection methods such as the use of the sweep net. In this study, all direct sampling methods of cherry foliage produced much lower numbers of leafhoppers than did sticky traps. In addition to a better cost: benefit ratio, trap samples provide a continuous estimate of activity between sampling dates. Sweep net samples, on the other hand, do not measure activity.

None of the sampling methods evaluated in this study provided absolute estimates of leafhopper density. The suction sampler (D-vac) might be useful for absolute estimates but its use for this purpose was not evaluated. Since direct foliar sampling methods yielded such low numbers of leafhoppers, we could not compare sticky trap catches to direct estimates of leafhopper density in cherry.

Sticky traps, or any sort of continuous trapping method are also subject to deficiencies other than the ones already noted. Vandalism or any factor such as rain, sprinkler irrigation, or high winds that caused lost or damaged sticky traps produced a loss of a sample that could not be replaced. On the other hand, a faulty sweep net or D-vac sample possibly could be repeated immediately or postponed.

The demonstrated effect of trap height on the species composition of sticky trap catches emphasizes the leafhopper activity is stratified not only with respect to host plant species but with respect to physical structure of plant growth as well. In this study, each leafhopper species had a characteristic distribution with respect to height, which seems to be usual for most insect species (Wolfenbarger, 1948). We conclude, for example,

the *A. grex* and *D. sonorus* are chiefly inhabitants of weedy surface vegetation, seldom venturing into the cherry canopy even when feeding directly beneath it. Catches of *C. montanus*, in contrast, were more evenly distributed among various trap heights. Although *C. montanus* was trapped in higher numbers near the ground compared to greater heights, its relative abundance in trap catches above 1 m is evidence that it is a canopy feeder to a much greater extent than any other known X-vector species in our study area. Our results from direct sampling of cherry foliage tends to confirm this conclusion.

This study documented that *C. montanus* is by far the most prevalent known X-vector in central California cherry orchards. Our results were similar in this respect to yellow sticky trap collections made in 1967-68 (Johannes Joos and Wallace P. Schraeder, pers. comm.) and to surveys in 1978 (Purcell, unpublished data). Previous reviews (Nielson, 1968; Gilmer and Blodgett, 1976) did not consider *C. montanus* important in the spread of X-disease. Because of its abundance and its activity in cherry, we consider *C. montanus* to be an important vector of the X-disease agent to cherry in central California.

3. Tests of leafhoppers species whose ability to transmit the X-disease agent is not known.

Attempts to transmit the X-disease agent from cherry (buckskin) to celery with *C. montanus* were not successful in 1977 on three occasions: early May, late June, and mid-August. In 1978 *C. montanus* reared in colonies at Berkeley did transmit to celery from cherry approached-grafted the previous summer with twigs from X-diseased cherry. The symptoms in celery of the recently field-collected strains of the X-disease were identical to those of a strain of peach yellow leafroll X-disease (PYLR) maintained at Berkeley for over twenty years via transmission by *C. montanus*. However, the strain from cherry was more virulent to celery, typically causing total collapse of the plant 6 - 8 weeks after inoculation, whereas PYLR-infected plants usually remained alive for over 12 weeks after inoculation.

Because tests of additional leafhopper species as vectors and additional plant species as hosts required readily available host plants for transmission tests, we used the PYLR strain of X-disease and attempted further recoveries of a strain of buckskin from cherry in 1978.

The leafhoppers *Scaphytopius nitridus* (De Long) and *Acinopterus angulatus*, *Macrosteles fascifrons*, *Amblysellus grex*, *Deltocephalus sonorus*, and *Exitianus exitiousus* were tested as vectors of X-disease. *S. nitridus* and *A. angulatus* successfully transmitted the XDA. Tests of the other species were negative.

METHODS

Leafhopper colonies -- Three species of leafhoppers used in the tests described here were maintained in a greenhouse at Berkeley. *Colladonus montanus* (Van Duzee) and *Scaphytopius nitridus* were maintained on celery (*Apium graveolens* L., 'Tall Utah 52-70'). *Acinopterus angulatus* was colonized on broadleafed plantain, *Plantago major* L., except when confined on celery test plants. All colonies were maintained free from exposure to X-diseased plants and periodically tested for noninfectivity by holding plants exposed to colonies for diagnosis.

Transmission test procedures. The peach yellow leaf roll (PYLR) strain of X-disease, was maintained in the greenhouse at Berkeley via transmission by *C. montanus*. Test plants were 3-4 week old celery seedlings grown in 7 cm pots. In one experiment, open pollinated 'Fay Elberta' peach seedlings (*Prunus persicae*) were used. Test plants were changed periodically as indicated in the results of each test.

Leafhoppers acquired the XD agent by caging colonies of early instar nymphs of each species on celery having well-developed symptoms of PYLR. Diseased source plants were changed as needed until the leafhoppers had spent at least 30 days on the source plants; in some tests acquisition feeding periods were shorter. Greenhouse temperatures during acquisition feeding periods averaged approximately 23°C. Transmission test plants were maintained in a continuous light growth chamber adjusted to maintain the temperature within cages at 25°C. Celery not exposed to leafhoppers served as controls. All plants were treated with dimethoate (Cygon® 25 WP) after leafhoppers were removed and held 10 weeks or longer for diagnosis for X-disease.

In one experiment, noninfectious late instar nymphs of *S. nitridus* and *C. montanus* were simultaneously injected with extracts of the XD agent prepared from 100 *C. montanus* that had fed for at least 30 days on PYLR-infected celery. The leafhoppers from which the extract was prepared were triturated in 1.5 ml of .01 M phosphate buffered 10% sucrose with 10,000 units/ml Penicillan G (Difco). This mixture was centrifuged at 10,000 rpm in a SS-34 rotor in a Sorvall model RC2-B centrifuge for 10 min (approx. 12,000 g). The supernatant then was filtered by syringe through a 0.45 M millipore filter. This extract was injected into CO_2-anesthetized leafhoppers via machine-pulled glass needles.

RESULTS

Feeding acquisition -- Transmission of the XD agent to celery by *S. nitridus* and *A. angulatus* following feeding on diseased source plants was relatively inefficient (Tables 3, 4). Only 11% (3/47) of *A. angulatus* transmitted in test no. 1 (Table 3). In a second test of *A. angulatus*, only 2 of 25 (8%) transmitted to the first test plant following acquisition feeding but not thereafter. Poor colony survival of *A. acinopterus* precluded further tests with this species.

In an initial transmission experiment, only one of 30 *S. nitridus* allowed acquisition feeding for 17 days transmitted the XD agent to the 7th

in a series of 13 approximately weekly transfers. At this time only 9 of the original group of 30 insects were alive. In a second test of S. nitridus, none of 22 tested insects transmitted after a 15 day feeding access. The results of a final test in which 5 of 26 S. nitridus transmitted, are shown in Table 4.

Transmission by S. nitridus after injection. Because transmission following feeding acquisition was inefficient and required a very lengthy latent period, S. nitridus 4th or 5th instar nymphs were injected with infectious extracts of XD agent to increase the number of transmitting insects and to reduce the required latent period. Noninfective C. montanus were also injected with the same undiluted extract for comparison. The transmission results are shown in Tables 5 and 6. Transmission to celery following injection was excellent for S. nitridus and C. montanus. Peach test plants were used as the 9th and final plants for injected S. nitridus in this sequence. One of 8 seedlings developed symptoms typical of PYLR beginning about 10 weeks after inoculation. This verified that our PYLR isolate of the XD agent remained pathogenic for peach.

DISCUSSION

The demonstration that S. nitridus and A. angulatus are potential vectors of X-disease adds to a growing list of proven vector species of the XD agent. Recent tests of additional leafhopper species X-vectors may have been restrained by an over reliance on celery. Other host plants of XDA which are more suitable for survival of leafhopper species that survive poorly on celery probably would increase the usefulness of both positive and negative results of tests of vector ability.

Infecting C. montanus by injection compared to feeding on diseased plants reduced the average latent period and increased the number of transmissions (Whitcomb et al., 1966). Injection may be useful in circumventing the difficulties of a very long latent period and inefficient transmission. Leafhopper species from newly established colonies undergoing laboratory transmission tests often survive poorly, particularly on a test plant selected primarily for its susceptibility to a particular disease agent rather than for its effect on leafhoppers. To facilitate transmission studies, candidate leafhoppers can be maintained after injection or feeding acquisition on the most suitable host plant for an interim period until they are caged on test plants. Feeding and survival of leafhoppers on test plants, however, still must be adequate for transmission to these test plants. Reduction of the latent period may facilitate identifying vector abilities in species that have inherently long latent periods and rather short life spans under laboratory conditions. In feeding acquisition experiments for example, the minimum latent period in S. nitridus of 76 days (Table 4) exceeded the average maximum lifespan of some lab-reared leafhoppers.

The greatly decreased latent periods produced by injection also strongly suggest that a "gut barrier" is a significant obstacle for the XD agent to overcome in S. nitridus. "Gut barrier" includes all possible events

from feeding uptake of the XD agent to the point where it begins to multiply in the leafhopper body cavity. Transmission by injected *S. nitridus* was significantly lower and the latent period much longer than that of injected *C. montanus*, which suggests that multiplication or passage through the salivary system of *S. nitridus* is less efficient than in *C. montanus*. The pattern of transmission of the XD agent whereby transmissions occur most often in consecutive sequences that end before individual vectors did is evident from these tests (Tables 4, 5). There is no evidence from my results, however, that transmission is negatively correlated with longevity in *S. nitridus*, as has been noted for *C. montanus* (Jensen et al., 1967). In fact, *S. nitridus* that transmitted in these trials had a greater average longevity than those that did not transmit in both feeding acquisition and needle-injection trials. The small number of transmitters, however, preclude meaningful statistical comparisons. More detailed studies on survival and fecundity are needed to assess the pathogenicity of the XD agent in *S. nitridus*.

Since neither *S. nitridus* or *A. angulatus* have been prominent in previous surveys (Jensen, 1956; Rice and Jones, 1972) of the leafhopper fauna of orchards or orchard vicinities in California affected by X-disease, their importance in the natural spread of X-disease in California is not likely to be great. Their importance in the dissemination of the XD agent to other plant hosts that are less noticeable as weeds or as components of uncultivated environments has not been assessed.

Similar efforts to test the competence of additional leafhopper species such as *A. grex* and *D. sonorus* that are numerically prominent in cherry orchards were unsucessful because of heavy mortality on celery or filaree test plants and rearing difficulties. Tests of these leafhoppers with recently field-collected isolates of X-disease are now in progress.

4. Biology of X-vector species.

Our sampling results indicated that C. montanus was the most abundant known vector of X-disease in cherry orchards. However, the density of this leafhopper in cherry orchards was quite low, so that it was difficult to make extensive field studies of host and habitat preferences, movements, and natural mortality agents.

In the Stockton-Lodi area, our surveys indicate that C. montanus has 3 generations per year, is probably quite active in its dispersal movements, has a wide range of plant hosts, and is most likely to feed on cherry in early autumn or late summer.

In 1978 one orchard in which a moderate number of C. montanus were recorded was more intensively sampled with sticky traps and sweep net samples in an attempt to gather data on the spatial distribution of the species in cherry. Such information would be useful in relating the spatial distribution of X-disease to that of C. montanus and in possibly providing clues as to the habitat requirements of this species.

Additionally, we tested the ability of C. montanus to acquire the XDA from cherry at various times during the growing season from mid-April to late September. The seasonal variation in acquisition rate might be important in determining when C. montanus is most likely to become infective with XDA.

Spatial distribution of C. montanus -- sticky trap sampling was continued in a 50 acre cherry orchard with the purpose of collecting data on the spatial distribution of C. montanus. The orchard, which was located 3 mi southeast of Lodi, CA, was divided into 16 equal blocks. Eight traps were placed in each of the four center and four corner blocks and monitored as in 1977.

The numbers and species of leafhoppers captured were similar to the previous year (1977), particularly known X-vectors. Colladonus montanus had a spatial distribution that was slightly aggregated and could be described as a negative binomial distribution with a "K" value of slightly greater than 1 (Table 7).

METHODS

In February, 1978, seventeen colonies of C. montanus were raised in the greenhouse for field acquisition access trials. Nymphs were caged on branches of cherry trees with X-disease for one week. Five such exposures were made at six-week intervals starting at bud break and terminating before leaf fall: 8 April, 20 May, 1 July, 11 August, and 22 September. Four 'Bing' cherry trees approximately 25-years old on Mazzard rootstock, diagnosed the previous year to be infected with X-disease, were selected for leafhopper acquisition experiments. To localize the leafhoppers, groups of seventy-five third instar nymphs were placed in nylon mesh sleeve cages

which covered approximately a 2 1/2 foot branch on the northeast side of each tree. The sleeve cages were slipped over a branch with the bottom opening of the cage wrapped around a piece of foam rubber which was wrapped around the branch. A hose clamp was then used to secure the foam rubber and nylon mesh to the branch. A carton containing the seventy-five leafhoppers was placed inside the sleeve cage and the upper opening of the cage was secured to the branch with bailing wire to prevent escape of the leafhoppers. After sealing the sleeve cage, the lid was removed from the carton to allow the leafhoppers access to the foliage. Two cages were placed on each tree, thus allowing 150 leafhoppers acquisition to each of four trees.

After one week's acquisition access feeding period, the eight caged limbs were cut below the hose clamp and returned to the lab where the leafhoppers were placed on eight, two-month old celery plants and set in a growth chamber at 24°C with constant light. After one week on the interim celery plant, thirty-five leafhoppers were removed from each of the eight celery plants and placed individually on one-month-old celery seedlings. The remaining leafhoppers were left on the intermediate plants and placed in the greenhouse for an eight-week period to allow for symptom development. If a leafhopper died during the first week on the individual celery plant, it was replaced by a remaining leafhopper from the intermediate plant.

The leafhoppers on the celery test plants were kept in the growth chamber at 24°C and transferred weekly until their death. After each transfer, the plants were sprayed with dimethoate (Cygon^{R} 25 WP) to kill any leafhopper eggs and placed in the greenhouse (approximately 25°C) for two to three months for symptom expression, then discarded.

RESULTS

No symptoms of X-disease developed on any of the five groups of intermediate celery plants, which would have resulted if the extrinsic incubation period had been less than 13 days. The average latent period was approximately 36 days. Table 8 shows the survivorship and percent transmission of the five groups. The first group of leafhoppers placed in the field on 8 April and retrieved on 15 April, 1978 did not transmit possibly due to the seasonality of the micro-organism in the field (Table 8). However, of the second group of leafhoppers, placed in the field on 20 May and returned to the lab on 27 May, 3.5% transmitted successfully to celery (Table 8). Symptoms developed approximately five to eight weeks after the leafhoppers had been placed on the indicator hosts. The third group of leafhoppers placed in the field on 1 July transmitted the X-disease agent to the host plant but at a slightly reduced transmission rate. Group four, placed in the field on 11 August, resumed the higher transmission rate with the most number of celery plants developing X-disease symptoms, peaking at 14.2% transmission. The percent of transmission remained high (10%) in the last group before leaf fall. Figure 3 illustrates this trend. Examining the seasonality of the X-disease agent in the field (Table 8) reveals that acquisition was highest on 11 August and 29 eptember, a time when the number of Colladonus montanus captured in sticky traps was at its peak.

5. Spatial distribution of X-disease.

In 1978 the same orchard in which leafhopper trapping had been conducted and which had a high incidence of X-disease was mapped for the spatial distribution of X-disease. It was hoped that the spatial pattern that might give some clue as to the nature of spread of the disease, as has been noted in the eastern U.S. in peach orchards located near choke cherries (Gilmer and Blodgett, 1976).

METHODS

Layout of orchard plots -- The experimental plot (Fig. 5) was a cherry orchard near Lodi, California. It consisted of 72 rows of trees spaced 18 feet apart. Each row consisted of 74 trees spaced 38 feet apart for blocks A, B, C, E, F, G and part of block H while trees in block D and part of block H were spaced 18 feet apart. The orchard was bordered on the north, south and partly on the east by vineyards, partly on the east by cherry and on the west by walnuts. The trees in blocks A, B, C, E, F, and G were approximately 30 years old grafted onto "Mazzard" root stocks. The trees in block D and mostly H were grafted high on "Maheleb" rootstock and were less than 15 years old. Weeds were periodically mowed; irrigations were applied by sprinklers. During the three years (1975-1978) preceeding the period of the study, insecticides were not applied to the experimental plot.

Mapping -- Each tree space in every block was visually diagnosed and each tree quadrant (NE, SE, SW, NW) given a severity rating of one to four. The criteria for each rating were:

Rating	Criteria
1	little or no primary growth
2	as 1 above but with slight fruit symptom
3	as 1 above with definite fruit symptom
4	as 3 above but with terminal dieback
X	dead, vacant, or replanted (< 10' tall)

Ratings of 3 or 4 were definitively diagnosed as infections of X-disease, whereas there could be some doubt in the case of a rating of 2, and considerable doubt in a rating of 1. Since the shape and size of fruits are important characters for diagnosis of X-disease, the mapping was done during the period just before harvest.

RESULTS

From the data gathered from mapping of X-disease, we computed several statistics to assess the tendency of X-disease to occur in aggregates of "clumps". All of these statistics estimated a low level of aggregation of X-disease.

1) Holgate's statistic: $h_B = \quad Xi^2 / \quad Wi^2$

2) Holgate's normal index: $h_n = m^{-1} \quad (X_i^2 / W_i^2)$

3) Index of dispersion: $I = \frac{Sx^2}{\bar{x}}$

4) Pielou's statistic (1959): $= D \bar{W}$

The values of the computed statistics and the values necessary to imply significant aggregation are given in Table 9. Figure 5 shows the actual distribution of various disease ratings in the mapped portions of the orchard. Both the appearance of the distribution of diseased trees and the statistical measures of aggregation suggest that X-disease is only slightly aggregated or random. Similar analyses for another orchard with less than 0.5% X-disease indicated that the disease was distributed randomly.

5. Common Weeds of Cherry Orchards and their Role as Alternate Hosts of the X-disease agent.

A listing of the most common weeds in or adjacent to cherry orchards was compiled from notes taken in 1977-78 in the 9 orchards during trapping surveys for leafhoppers (Table 10).

Several species from this list were grown from seed in the greenhouse at Berkeley in 1977 for testing as hosts of X-disease agent. As field-collected isolates of XDA were not available, PYLR in celery served as the inoculum source. Early-instar C. montanus nymphs were placed on celery with advanced symptoms of PYLR for 30 days and then transferred singly to small celery seedlings to test their infectivity. After 3 - 7 days, the insects were transferred to weed test plants either singly or in groups as indicated in Table 11. The test plants were saved for 3 - 4 months for diagnosis for X-disease. Some apparently infected plants were then tested for the presence of XDA by caging non-infective C. montanus on them for one week, and then transferring them to healthy celery to check for transmission.

Our findings extend the herbaceous host range of PYLR established largely by Jensen (1971). Because of the extreme abundance of such weeds as filaree, plantain, fiddleneck, and shepherd's purse in and around cherry orchards, further understanding of the infection process in these and other weed hosts is important to our knowledge of the epidemiology of X-disease. Apparently, PYLR has a very wide plant host range. This will be confirmed for a field strain recently collected from cherry.

An interesting preliminary finding was that the age of some plant species may have an extreme effect on susceptibility to infection by the XDA. We attempted on three separate occasions to inoculate approximately 2 - 3 month old filaree plants with two or three leaf cages per plant with 10 - 12 inoculative C. montanus per cage. None of these efforts, using six plants per attempt, were successful. On the last such attempt, some of the inoculative leafhoppers were caged singly on small filaree seedlings and 5 out of 12 became infected. The effect of plant age on susceptibility to X-disease may in part explain the low incidence of infected weeds in natural vegetation in orchards with a high incidence of X-diseased trees.

B. Diagnosis and Etiology of X-disease in Cherry

1. Diagnosis

Accurate diagnosis of XD in cherry is very difficult except when mature fruit is on the tree and then only with certain scion-rootstock combinations. More reliable diagnostic techniques would aid in distinguishing trees that have XD from those afflicted with other disorders.

The following diagnostic methods were evaluated: (1) histological techniques, (2) electron microscopy (EM) -- both scanning and transmission EM, and (3) serodiagnostic methods.

Histological Techniques -- Acidified methanol and phloroglucinol were evaluated as histological stains to detect differences between healthy and X-infected cherry in the field. Bark samples near the bud union were taken from healthy and X-diseased trees in August, after fruiting. Samples were placed into vials for 3-5 minutes and rated according to color differences. Samples of X-diseased and healthy bark samples reacted similarly in both acidified methanol and phloroglucinol. These tests were not pursued further because of the uniformly negative initial results.

Scanning Electron Microscopy (SEM) -- It is a standard technique to use transmission electron microscopy (TEM) to show the presence of mycoplasma-like organisms in the phloem of X-infected tissues. The methods used for preparation of plant materials for TEM are time consuming and rather expensive. However, samples can be prepared for scanning electron microscopy (SEM) in a few hours.

To investigate the use of SEM for diagnosis of XD, we examined pedicels and petioles from X-infected cherry and celery using standard techniques. MLO were observed in phloem elements appressed to sieve plates of X-infected cherry and celery. Similar MLO-like structures could be observed in the phloem of healthy plants, although these structures were generally larger, of smaller density, and were not appressed to sieve plates, as in the infected material. The highest quality materials were obtained with cryofracture of fresh fixed samples followed by lyophilization. Although the presence of bleb-like structures in healthy plants presents a problem in diagnosis by SEM, the principle difficulty encountered was the extremely low number of sieve elements containing MLO detected per petiole fracture plane. This study did show that the MLO's in X-infected plants vary greatly in morphology and size. No spiral forms were present, but some MLO were filamentous.

Seasonal Occurrence of Mycoplasma-Like Organisms in X-Infected Cherry

Transmission electron microscopy was used to determine chronologically when the MLO were present in infected tissues. This information might aid in knowing when to attempt isolation of the MLO and also might suggest when the MLO could be most readily obtained by leafhopper vectors.

Leaves and pedicels from infected and healthy cherry trees were sampled from early bloom to post harvest. Tissues were transported on ice and fixed in Osmium tetroxide-glutaraldehyde within 12 hours. Material was embedded in Spurr's resin and thin sections were made with an ultramicrotome. Samples were viewed with an RCA EMU-3 electron microscope.

No MLOs were observed in petioles or pedicels until symptoms were apparent on the trees, although MLO's were found in symptomless branches of infected trees. We first observed MLO in pedicels in low numbers and in each succeeding sample the number of MLO increased. MLO were observed in petioles about two weeks after they were first observed in pedicels and also increased in concentration until entire phloem elements were completely full of MLO.

Serodiagnostic Techniques: Immunodiffusion, Immunoelectrophoresis, ELISA --

METHODS

Serological methods have been developed for rapid diagnosis of several plant diseases using antisera prepared against pure cultures of the causal organism or purified or concentrated virus preparations. Since the X organism had not been cultured or purified, we initially attempted to produce antisera to the X-organism by using extracts of diseased plant and leafhopper tissues as antigens.

Antisera were produced in rabbits using standard injection schedules. Antigen preparations were made from the vascular tissues of X-diseased celery plants and the entire bodies of X-diseased Colladonus montanus leafhoppers. Gamma globulin fractions were separated from whole serum by ammonium sulfate precipitation. Antisera against uninfected tissues were produced and treated in the same manner.

A number of spiroplasma isolates were obtained from Dr. B. C. Raju and Dr. George Nyland, Dept. of Plant Pathology, University of California, Davis. These isolates were cultured from different diseased plants as listed in Table 1. Also listed in Table 1 are other spiroplasma isolates received from other laboratories. Thus a number of different spiroplasma isolates were available for comparison using various serological tests.

Antigens from X-diseased plants were prepared from 150 g of celery tissue or 70 g of X-celery vascular bundles ground in approximately 200 ml glycine extraction buffer (Nasu, et al., 1970) in a blender. This preparation was pelleted to clarify at 10,000 G and the supernatant centrifuged for 1 hr at 65,000 G. The resulting high speed pellet was suspended in 10 ml of the extraction buffer. In one test, the high speed centrifugation was at 100,000 G for 1 hr. For double-diffusion tests, about 150 g was ground in 30 ml of glycine extraction buffer in a blender. The resulting slurry was filtered through several layers of cheesecloth and the slow speed pellet

(10,000 G) removed. The resulting supernatant was used as an antigen preparation in double diffusion tests. Leafhopper antigens were prepared in a similar manner except that 0.5 g of leafhoppers were ground in 8 ml of glycine buffer or .01 phosphate buffered saline and the slow-speed pellet reground, resuspended, and centrifuged again so as to maximize the concentration of mycoplasma. The high speed centrifugation of leafhopper extracts was made at 100,000 G in 0.15 M phosphate-buffered 10% sucrose.

Spiroplasma antigens were prepared from late log phase growth in 1 to 2 liters of broth media. These were pelleted by centrifugation. Membrane antigens used in immunoelectrophoresis tests were osmotically lyzed in water and sonicated to release cytoplasmic antigens. The membranes were washed several times with phosphate buffer to remove soluble cytoplasmic antigens and the solubilized with detergents.

Preliminary tests of ELISA used antiserum produced against the spiroplasma isolate SX (Table 12). After purification, the antiserum was conjugated with an alkaline phosphatase, and tests were performed using the system developed by Clarke and Adams (1976). The antiserum was initially adjusted to a protein concentration of approximately 1 mg/ml.

RESULTS

The antisera produced against X-diseased celery reacted strongly in gel double diffusion tests with extracts from X-diseased _C. montanus_ leafhopper extracts but did not react against healthy leafhopper extracts (Table 13). This antiserum also reacted with X-diseased celery and healthy celery extracts, but spur formation (Figure 6) indicated the presence of additional, unshared components in X-diseased celery compared with healthy celery. To attempt to identify these additional components, leafhoppers fed on 10 plants for 30 days were placed on X-diseased celery plants and healthy celery plants for 1 week. When tested in gel double diffusion against anti-XC antiserum, the X-leafhoppers from the healthy plant did _not_ react while the X-leafhoppers continually fed on X-diseased celery reacted in the usual manner. We thereby conclude that these reactions between the anti-XC and various antigens may be a protein associated with X-diseased plant material and not the X mycoplasma (see Figure 6). Antisera produced against X-diseased leafhoppers react with both diseased and healthy leafhoppers.

The anti-X-disease celery (anti-XC) antiserum was tested in gel double diffusion against all known and unknown spiroplasma isolates (Table 13) and no reactions between any spiroplasma and anti-XC were observed.

Rocket immunoelectrophoresis tests using 6 antigens in each run were made for each of the antisera listed in Table 14. Two broad groupings of spiroplasma isolates were apparent, as shown in Table 14. One group consisted of _Spiroplasma citri_, the SC isolate from PYLR celery, and an isolate from _C. montanus_ fed on PYLR celery. The Morocco isolate (Dr. E. C. Calavan,

U.C., Riverside) of S. citri, interestingly, did not react similarly to other strains of S. citri. The second grouping which showed many common antigens were three aster yellow isolates, a PYLR isolate from peach, X-diseased cherry, pear with pear decline, the honeybee spiroplasma (AS 576), and a spiroplasma isolated from flowers (GI). These tests were unable to clearly differentiate any of the isolates within each major grouping.

The antiserum produced against diseased celery (peach yellow leaf roll) extracts was cross absorbed against healthy celery to remove some of the cross-reacting components. This absorbed antiserum reacted forming a strong precipitin band in immunoelectrophoresis when extracts from infectious leafhoppers (C. montanus) but not healthy leafhoppers were tested. No reaction was obtained when soluble membrane antigens from the SX, CBS, SGV, PYLR, and SC-B or whole cell freeze-thaw preparations of the same antigens were tested. It would seem that if common antigens existed between the diseased celery extract and any of the spiroplasmas suspected of causing the disease precipitin bands would have been observed at least against some of the spiroplasma strains since a reaction was observed against infectious but not healthy leafhopper extracts.

ELISA -- The first series of tests was designed to evaluate the sensitivity of the homologous reaction of conjugated enzyme/antiserum. The results were positive at concentrations of conjugated antiserum diluted 1:750 and the coating gamma-globulin diluted 1:200. The SC antigen (Table 2) was an initially concentrated (100X) sample which was then diluted 1:3750. This was equivalent to a 38-fold dilution of a culture of the SX organism. The antigen had been frozen and thawed. A color reaction at all concentrations of the reactant indicated a strong positive reaction. The intensity of the reaction correlated with the concentration of antigen and conjugated antiserum reaching near endpoint, i.e. the color was very faint, at the highest dilutions (Table 15).

A second experiment tested specificity of the reaction of SX antiserum against: 1) an isolate of Spiroplasma citri obtained by isolation from a plant in our greenhouse known to be infected by S. citri, 2) a spiroplasma isolate obtained by Dr. B. C. Raju from a plant with symptoms of aster yellows disease, 3) tobacco mosaic virus, 4) the homologous SX organism.

Antisera were diluted to a maximum of 1:200. The spiroplasma antigens were frozen, concentrated 100X, and diluted to a maximum of 1:400 for the test. This corresponds to a 4- fold dilution from a standard culture. The TMV was initially 10 mg virus/ml diluted to a maximum of 1:4000 (2.5×10^{-3} mg/ml).

The SX and S. citri antigens reacted identically in the test with absorbances greater than 2.0. The aster yellows isolate reacted, but the intensity of the reaction -- about 0.09 absorbance -- was reduced greatly in comparison to the SX and S. citri reactions. TMV did not react

at all (no color was produced).

The tests indicate ELISA may be useful for diagnosis of spiroplasmal diseases. However, the SX isolate has not been demonstrated to be the pathogen inciting X-disease of cherries. The continuing attempt to diagnose X-disease in orchard trees will involve conjugation and testing of various antisera produced against spiroplasmas associated with X-diseased plants.

2. Culture of the X-Agent

The inability to culture the causal organism on artificial media is a deterrent to research and subsequent control of X-disease. Without culturing, most research is dependent on time-consuming methods of grafting or insect transmission. If the organism could be cultured, it should be possible to rapidly and reliably diagnose infected plants. Candidate chemicals for control of X-disease could also be rapidly assayed by using in-vitro tests. Antiserum produced against the cultured organism would have high specific titer and could be used in some of the highly sensitive serodiagnostic techniques for rapid field diagnosis.

METHODS

Media formulations were patterned after media developed for culture of *Spiroplasma citri* and other spiroplasmas (Table 16). The sources of inoculum were X-diseased celery grown in the greenhouse, X-diseased *C. montanus* leafhoppers, X-diseased cherry, and other plants infected with X disease (lettuce and plantago).

Infected tissues were surface sterilized in 1 percent NaOCl and rinsed 3 times in sterile distilled water. They were then chopped with a razor blade in 5 mls. of the liquid isolation medium (#1). The resultant material was filtered through an 0.45 um membrane filter, and 1 ml of this inoculum was added to 4 ml of the isolation medium in glass screw cap tubes. Tubes were incubated tightly capped at 28° in the dark. Blind transfers of the cultures were made on days 1, 4, 7 and 14. Aliquots were removed periodically for examination under darkfield microscopy. An indication of growth was taken to be 1) a change in the media color from red to yellow, indicating a pH shift from slightly basic to acidic, or 2) an increase in the number of particles visible under darkfield microscope examination.

RESULTS

A helical motile spiroplasma was isolated in one instance (out of 50 attempts using both healthy and diseased plants) from X-diseased celery (peach yellow leafroll strain). This spiroplasma isolate was successfully subcultured in a simplified maintenance medium (reduced horse serum and fresh yeast extract) at passage dilutions of 1:100 to produce high spiroplasma concentrations in 2 - 3 days. Colonies of the spiroplasma on solid media (1% noble agar) are of fried egg and granular types. In many of the isolation

attempts, pleiomorphic blebs could be observed at low levels, but these structures never reached high concentrations and growth diminished through successive passages. Concurrent with the isolation of this spiroplasma from peach yellow leafroll (PYLR) infected celery tissue in our laboratory, Dr. B. C. Raju of U.C. Davis, Dept. of Plant Pathology, isolated spiroplasma from the same plant source using identical isolation procedures and a modified isolation media (Table 2: Isolation Media #2). He has since been able to isolate spiroplasmas from a wide range of infected plant sources with a high frequency of positive isolations. Our attempts to duplicate his isolations using this modified media have produced negative results.

Through the cooperation of Dr. Raju, over 20 spiroplasma isolates from different hosts and diseases were maintained in our laboratory. These spiroplasmas were used as a tool to undertake a second approach to primary culture of the X-disease organisms. We found that the PYLR celery spiroplasma (SX) survived and multiplied in the haemolymph of greased wax moth (*Galleria mellonella*) larvae, as did other spiroplasma including S. citri, corn stunt spiroplasma and AS 576 (honeybee spiroplasma). Ten microliters of log phase spiroplasma culture were injected through the proleg of *G. mellonella* larvae. After 10 days the haemolymph was collected and examined under darkfield nicroscopy, where extremely high concentrations of active spiroplasma were observed. Using this system, *G. mellonella* larvae were injected with extracts of X-diseased *C. montanus* leafhoppers, producing one positive isolation of spiroplasma out of 40 larvae injected. The spiroplasma isolated in this manner exhibited extremely rapid growth in medium #2 (compared to SX and other isolates) and produced granular colonies on solid medium.

Attempts to transmit the cultured SC spiroplasma to celery by injecting noninfective *C. montanus* leafhoppers were made on several occasions with no transmissions. A number of X-isolates from Drs. B. C. Raju and G. Nyland at U.C. Davis were similarly injected into *C. montanus*. Usually 80 to 150 insects were injected with each isolate and then confined on test plants. The test plants were then kept for 10 weeks or longer. No transmissions of XDA were noted except for leafhoppers injected with fresh extracts prepared from macerated infective leafhoppers. This lack of transmission suggests that the spiroplasma cultured from X-diseased plants is either not the causal organism or looses pathogenicity in culture.

SUMMARY OF MAJOR FINDINGS AND CONCLUSIONS

1. The species composition of leafhoppers present in cherry foliage varies dramatically from that found in orchard cover vegetation.

2. Sampling methods that detect relative leafhopper abundance and activity over prolonged periods seem most applicable to studies of leafhoppers relative to their possible roles as vectors of X-disease in cherry. Of the sampling methods tested in this study, yellow sticky traps were most appropriate for this purpose.

3. Orchard weed control practices, i.e. cultivation or sod culture, seem to have little effect on the leafhoppers detected in cherry trees.

4. Colladonus montanus was by far the most abundant known vector of X-disease present in cherry orchard surveys. This leafhopper species was also relatively active on cherry foliage and was the only species recovered directly from cherry foliage. Colladonus montanus is probably the most important vector of X-disease to cherry in central California.

5. The leafhoppers Acinopterus angulatus and Scaphytopius nitridus were proven to be vectors of X-disease. Their importance in natural spread of X-disease in California is minor.

6. Colladonus montanus has 3 generations per year in central California. It is a relatively active flier and is rarely aggregated or clustered in its spatial distribution.

7. The seasonal availability of the X-disease agent in cherry for leafhopper acquisition by feeding ranged from a very low potential (zero in our tests) in April to a peak of 14% in August with a slight decline in September.

8. X-disease occurred either at random or in loose "clusters" within cherry orchards surveyed.

9. Weeds that are quite common in most cherry orchards in San Joaquin Co., CA can be infected with the peach yellow leafroll strain of the X-disease agent. Examples are filaree, mustard, fiddleneck, and plantain.

10. Scanning electron microscopy can be used to detect mycoplasma-like organisms in tissues of cherry with X-disease. This approach is not feasible for rapid diagnosis of large numbers of plants.

11. Serological tests of spiroplasma isolated from plants with X-disease indicated that these isolates share many common antigens with each other and with spiroplasmas isolated from bees and flowers.

12. Attempts to prove that spiroplasma and mycoplasma-like organisms isolated from X-diseased plants are in fact the causal agent have been unsuccessful.

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Table 1. Leafhoppers collected by sweep net, D-vac or yellow sticky traps in a clean-cultivated (disked) and mowed orchard. San Joaquin Co., CA. 1977[a].

Species (* = known X-vector)	Sample Method[a] Sweep		D-vac		Yellow sticky trap	
	mowed (n=175)	disked (n=93)	mowed (n=65)	disked (n=31)	mowed (n=172)	disked (n=169)
Aceratagallia spp.	78	57	0	8	1	2
**Acinopterus angulatus*	8	0	1	0	1	0
Amblysellus grex	1791	541	62	112	12	11
**Colladonus geminatus*	0	0	0	0	1	4
**Colladonus montanus*	26	13	4	3	82	19
Deltocephalus sonorus	623	34	43	5	7	5
Draeculacephala minerva	0	0	0	0	1	1
Empoasca, *Dikrella*, *Balclutha*, *Edwardsiana*	791	383	38	353	347	1473
Erythroneura elegantula	6	0	12	1	67	1
**Euscelidius variegatus*	1	6	0	0	2	3
Exitianus exitiousus	453	352	0	9	1	9
**Fieberiella florii*	0	0	0	0	0	5
**Gyponana angulata*	0	0	0	0	3	0
Idiocerus spp.	0	0	0	0	1	1
Macrosteles fascifrons (Stål)	379	143	23	107	17	38
**Osbornellus borealis*	0	0	0	0	0	1
**Paraphlepsius* spp.	0	0	0	0	1	0

Table 1. (Con't)

[a] Eight samples of each method were taken weekly or biweekly. A sweep net sample was 50 sweeps of surface vegetation; D-vac samples were 25 sucks; a single 5 x 10 in. yellow sticky board coated on both sides and hung in the cherry canopy was a single trap sample.

[b] n = total no. of valid samples for each sample method for each location.

Table 2. Average number of leafhoppers collected weekly on yellow sticky traps in four mowed and five disked cherry orchards. San Joaquin Co., 1977.[a]

Species (* = known X-vector)	Average no./trap/week Type of weed control mowed (n=578)	 disked (n=774)	No.of orchards in which found
Aceratagallia spp.	(1)[b]	.06	5 (56%)
*Acinopterus angulatus	(1)	0	1
Amblysellus grex	.04	.06	9 (100%)
Circulifer tenellus	0	(3)	2
*Colladonus geminatus	(1)	.02	6
*C. montanus	.23	.34	9
Deltocephalus sonorus	.03	.03	8
Draeculacephala minerva	(1)	(1)	2
Empoasca, Dikrella, Balclutha, Edwardsiana spp.	5.00	6.35	9
Erythroneura elegantula	1.60	.68	8
*Eucelidius variegatus	.01	.02	6
Exitianus exitiosus	.01	.04	8
*Fieberiella florii	0	.02	4
*Gyponana angulata	.01	.02	4
Idiocerus spp.	.01	.01	6
Macrosteles fascifrons	.09	.18	9
*Osbornellus borealis	0	(1)	1
*Paraphlepsius spp.	(2)	(2)	3
*Scaphytopius spp.	0	(3)	1
Unidentified	.01	.03	8

Table 2 (Con't)

[a] Eight sticky boards per orchard from Julian dates 109 to 298, 1977.

[b] Numbers in parenthesis indicate total number trapped ($\bar{x} < < .01$).

Table 3. Transmission of X-disease agent to celery by *Acinopterus angulatus*[a].

Insect No.[b]	First Day on Test Plant[a]				
	31	38	45	53	59
1.	+[c]			+	
2.	+			Dead	
3.	+			Dead	
4-5.					Dead
6-13.				Dead	
14-18.			Dead		
19-27.		Dead			

a. Late instar nymphs placed on diseased source plants (peach yellow leaf roll strain) for 30 days, then transferred to celery test plants on day 31.

b. Insects assigned number in order of (i) first and (ii) longest consecutive transmission.

c. + indicates test plant had symptoms of X-disease.

Table 4. Transmission of X-disease agent to celery by *Scaphytopius nitridus*[a].

Insect No.[b]	First Day on Test Plant[c]									
	51	62	70	76	86	93	100	106	113	119[d]
1.				+[c]	+	+	+		+	
2.				+	+	+		+		Dead
3.					+	+				
4.					+			+		
5.						+				
6-8.										
9.										Dead
10-13.									Dead	
14.							Dead			
15-16.						Dead				
17-19.					Dead					
20-21.				Dead						
22-23.			Dead							
24-26.		Dead								

a. Nymphs placed on diseased source plants for 30 days then transferred to celery test plants on day 31.

b. Insects arranged here in order of their first transmissions or longevity.

c. + indicates test plant had symptoms of X-disease. Test plants up to "day 51" were not infected.

d. Insects no. 1, 6, 7, and 8 lived until day no. 155 without transmitting.

Table 5. Transmission of X-disease agent to celery by *Scaphytopius nitridus* following needle injection of extracts[a].

Insect no.[b]	First Day on Test Plant[c]							
	13	27	34	47	56	69	76	86
1.	+[d]	+	+	+	+	+	+	+
2-3.	+	+	+					Dead
4.	+	+						Dead
5.	+						Dead	
6.	+				Dead			
7.		+	+	+	+			
8.		+	+	+	+		Dead	
9.		+	+	+		+	+	+
10.		+	+	+				
11.			+	+	+	+		
12-13.			+	+	+			
14.			+	+		+		
15.			+	+		+	Dead	
16-17.			+				Dead	
18.					Dead			
19.				+	+	+		
20.				+	+			
21.				+				
22.						+		
23.							+	+
24.							+	e
25.								

Table 5 (Con't)

Insect no.[b]	First Day on Test Plant[c]							
	13	27	34	47	56	69	76	86
26.							Dead	----
27.					Dead	----	----	----
28.			Dead	----	----	----	----	----
29-30.		Dead	----	----	----	----	----	----

a. Late instar nymphs injected with the supernatant of an extract prepared from 100 macerated Colladonus montanus that had fed for over 30 days on diseased celery.

b. Insects numbered in order of first transmission or longevity.

c. Injected insects were pooled on a single plant for 12 days after injection, then transferred individually to celery test plants on day 13.

d. + indicates test plant had symptoms of X-disease. The ninth series of test plants were peach seedlings, one of which developed X-disease.

e. Two insects (nos. 24, 25) survived until day 150. Insect no. 24 transmitted once more on day 100-107; no. 25 never transmitted.

Table 6. Comparison of transmission of X-disease agent to celery by *Scaphytopius nitridus* (Sn) and *Colladonus montanus* (Cm) following feeding on diseased course plants or needle injection of infectious extracts[a].

	% Transmission[b]	LP_{50}[c] (days)	LP_{min}[d]
Sn fed	19% (38%)	79	46
Cm fed	78% (89%)	28	19
Sn injected	77% (94%)	34	13*(20)
Cm injected	100% (100%)	18	13

a. Extracts of XD agent prepared using 100 infectious Cm macerated in 1.5 ml of .01 m phosphate buffered 10% sucrose.

b. Percentage of inoculated insects that transmitted to celery test plants. Figures in parentheses include only insects that survived to the date of the first transmission by the insect that was the last to begin transmit

c. Median latent period (LP_{50}) estimated by linear regression of log time and percent transmission.

d. Minimum latent period (LP_{min}) is the shortest time that any insect in group indicated could have transmitted, including time spent (if any) on diseased source plants.

Table 7. Value of exponent (K) of negative binomial distribution of sticky trap catches of *Colladonus montanus*.

Date of sample	Total catch	K[a]
	Systematic (non-random) sampling[b]	
7-27-78	26	1.10
10-12-78	17	1.07
8-31-78	4	1.01
entire season, 1978	168	0.03
	Random sampling[b]	
7-27-78	16	1.24
10-12-78	13	1.24
8-31-78	4	1.04

a. Calculated for $\log N/No = K \log (1 + \frac{\bar{x}}{k})$ where N = No. of samples, No = number of samples of zero; $\bar{x}$ = average sample size.

b. Systematic samples were evenly spaced; "random" samples were a random selection of systematic samples.

Table 8. Seasonal transmission of X-disease agent from cherry to celery by *Colladonus montanus*.

Acquisition feeding period on cherry:	Apr. 8-15	May 20-27	July 1-8	Aug. 11-18	Sept. 22-29
Tree no. (Limb)					
1 (A)	0	0	2/35[a]	10/33	9/35
(B)	0	0	0	6/35	8/35
2 (A)	0	6/35	2/35	2/35	2/35
(B)	0	3/34	0	0	-[b]
3 (A)	0	2/35	3/35	11/35	4/35
(B)	0	1/22	5/35	8/31	-
4 (A)	0	0/35	0	15/35	8/35
(B)	0	2/35	0	11/32	-

[a] Numerator is number that transmitted to celery; denominator is number surviving.

[b] Not done.

Table 9. Spatial distribution indices of X-diseased cherry trees.

Statistic[a]	Observed value	Minimum value for aggregation (p 5%)
1. Holgate's beta	0.58	0.64
2. Holgate's Z	1.16	1.65
3. Dispersion index	34.03	36.42
4. Pielou's alpha	15.11	19.68

[a] References: Holgate's beta and Z -- Holgate (1955); dispersion index -- Fischer, et al. (1922); Pielou's alpha -- Pielou (1959).

Table 10. Common weeds in cherry orchards, Stockton-Lodi, CA 1977-78.

Spring-summer annuals

forbs

purslane *Portulaca oleracea* L.
pigweed *Amaranthus hybridus* L. and *A. retroflexus* L.
Sowthistle *Sonchus oleraceus* L.
prickly lettuce *Lactuca scariola* L.
lamb's quarters *Chenopodium album* L.
black nightshade *Solanum nodiflorum* Jacq.
dandelion *Taraxacum officinale* Weber
plantain *Plantago lanceolata* L.
knotweed *Polygonum avicutare* L.
cat's ear *Hypochoeri glabra* L.
spurge *Euphorbia maculata* L.

grasses

crabgrass *Digitaria sanguinalis* L.
water grass *Echinochloa crusgalli* (L.) Beav.
rescue grass *Bromus willdenowill* Kunth.
foxtail *Setaria verticillata* Beauv., *S. glauca* (L.)
lovegrass *Eragrostis diffusa* Buckl.
cupgrass *Eriochloa gracilis* (Fourn.) Hitchc.

Winter and Spring annuals

forbs

chickweed Stellaria media (L.)
groundsel *Senecio vulgaris* L.
filaree *Erodium cicutarium* (L.)
pineapple weed *Matricaria matricarioides* (Less.)
black mustard *Brassica nigra* (L.)
fiddleneck *Amsinkia intermedia* F. & M.
shepherd's purse *Capsella bursa-pastoris* (L.)
miner's lettuce *Montia perfoliata*
nettle *Urtica urens* L.
cheeseweed *Malva parviflora* L.
henbit *Lamium amplexicaule* L.
dock *Rumex crispus* L.
red maids *Callandrinia ciliata* (R. & P.) DC.
milk thistle *Silybum marianum*
London rocket *Sisymbrium irio* L.
speedwell *Veronica arvensis* L.
willow-herb *Epilobium paniculatum* Natt.
popcorn flower *Plagiobothrys canescens* Beuth.

Table 10. Con't)

grasses

ripgut brome	*Bromus rigidus* Roth.
soft chess	*Bromus mollis* L.
wild oats	*Avena fatua* L.
wild barley	*Hordeum leporinum* Link.
annual bluegrass	*Poa annua* L.

Perennials

bermudagrass	*Cynodon dactylon* L.
dallisgrass	*Paspalum dilatatum* Poir.
burclover	*Medicago polymorpha* L.
bindweed	*Convolvulus arverusis* L.

Table 11. Transmission of X-disease agent to herbaceous plants.

Host	Transmission Results			
	No. plants tested[a]	Av.No.of Cm/plant	Inoculation period (days)	Positive controls (celery)
Dock	0/11	3	8	1/1 (8/10)
Fennel	0/8	2	8	2/2
Hemlock	0/6	2	8	10/12
Cheeseweed	0/5	2	8	4/4
Grounsel	0/4	1	7	6/10
Shepherd's purse	2/18	1	7	2/3
Filaree (red-stem)	3/30	1	4	15/30
Henbit	0/17	1	4	19/35
Watercress	0/26	1	3	13/26
Fiddleneck	2/6	1	8	4/6
Radish	7/12	1	8	-
Dandelion	1/15	1	6	2/5
Willow Herb	0/5	1	4	12/17
Bindweed	0/5	10	7	-
Cheeseweed (2nd trial)	0/6	10	12	5/6
Plantain (Plantago major)	4/6	10	7	6/6
Milkweed	0/13	1	7	-

[a] Numerator is number of positive transmissions of X-disease agent; denominator is number of test plants on which leafhoppers survived.

Table 12. Source of spiroplasmas including those isolated in this study

Spiroplasma	Strain	Source	Isolation Host
Spiroplasma Citri	189	(1)	Citrus with stubborn disease
"	Morrocco	(1)	"
"	MC 909	(1)	Unknown
"	W 762	(1)	Unknown
"	SC-B	(3)	Citrus with stubborn disease
Honey bee spiroplasma	AS 576	(2)	Honeybee
Tulip flower spiroplasma			
	G-1	(2)	Tulip tree flower
Unknown	SX	(3)	Celery with peach yellow leaf roll strain of Western X
"	WXCM	(4)	<u>Colladonus</u> <u>montanus</u> leafhopper fed on PYLR strain infected celery
"	TLAY	(4)	Plant with Tulee Lake strain of Aster Yellows
"	CBS	(4)	Western-X diseased cherry tree
"	PYLR	(4)	Peach tree with peach yellow leafroll
"	DAY	(4)	Plant with dwarf aster yellows
"	AYG	(4)	Plant with unknown strain of aster yellows
"	PD	(4)	Pear with pear decline
"	XGV	(4)	Celery with Green Valley strain of Western-X
Corn stunt spiroplasma	747	(4)	Corn with corn stunt disease

Sources: (1) E.C. Calavan, Univ. of Calif., Riverside; (2) R. E. Davis, USDA, Beltsville, MD.; (3) D. G. Garrott and S. V. Thomson, Univ. of Calif., Berkeley; (4) B. C. Raju and G. Nyland, Univ. of Calif., Davis.

Table 13. Reactions on gel double diffusion of antisera and extracts of X-diseased and healthy plant and leafhopper tissues.

Antisera	Extract							
	X Celery	X Cherry	X Leafhopper	Celery	Cherry	Leafhopper	X Leafhopper fed on X celery	X Leafhopper fed on H celery
X Celery	+	-	+	+	-	-	+	-
X Cherry	+	+	+	-	+	=	N.D.	N.D.
X Leafhopper	-	-	+	-	-	+	+	+
Celery	+	-	-	+	-	-	N.D.	N.D.
Cherry	-	+	-	-	+	-	N.D.	N.D.
Leafhopper	-	-	+	-	-	+	+	+

+ = precipitin band formation

- = no band formed

N.D. = not done

Table 14. COMPARISON OF SPIROPLASMA MEMBRANE ANTIGENS USING ROCKET IMMUNOELECTROPHORESIS

MEMBRANE ANTIGENS	ANTISERUM								
	SCB	SX	CS	CMWX	CBS	PYLR	DAY	PD	AS 576
SC-B	1[a](2)[b]	2(2)	1(2)	1(3)	(4)	(5)	(5)	(5)	(1)
SC 189	1(1)	2(2)	1(1)	1(1)	(3)	(3)	(3)	(3)	(1)
SC MC909	1(1)	2(2)	1(1)	1(2)	(3)	(1)	(4)	(3)	(2)
SC W762	1(1)	2(2)	1(1)	1(1)	(3)	(2)	(4)	(3)	(2)
SX	1(2)	2(2)	1(2)	1(4)	(6)	(4)	(5)	(1)	(1)
WXCM	1(2)	2(2)	1(1)	1(3)	(4)	(2)	(3)	(1)	(1)
SC MOROCCO					1(6)	2(2)	1(5)	2(2)	2(2)
TLAY					1(4)	2(4)	1(4)	2(2)	1(4)
CBS	(2)				2(6)	2(4)	1(7)	2(2)	1(4)
PYLR	(2)				2(4)	2(4)	1(5)	2(2)	1(4)
DAY					2(5)	1(5)	1(5)	2(2)	1(2)
AYG					2(5)	1(3)	1(4)	2(2)	1(2)
PD					1(7)	1(3)	1	2(2)	2(2)
AS 576					1(3)	1(4)	1(7)	2(2)	2(3)
G 1					1(2)	1(3)	1	2(2)	2(2)

[a] MAJOR PEAKS SEEN BEFORE STAINING.

[b] MINOR PEAKS SEEN AFTER STAINING.

Table 15. Ultraviolet absorbance of ELISA tests at varying dilutions of coating antisera and test antisera.

SX G-globulin (coating)	Test antigen dilution						
	Dilution	1:5	1:25	1:125	1:250	1:750	1:3750
	1:5[a]	2.65	2.62	2.61	2.32	1.27	0.27
	1:25[b]	2.56	2.52	0.19	1.32	0.49	0.04
	1:125[b]	2.62	2.46	0.20	1.64	0.74	0.03
	1:250[b]	2.51	2.38	0.14	1.36	0.95	0.08
	1:750[b]	2.09	2.02	0.26	0.29	0.06	-

a. Enzyme-conjugated SX G-globulin diluted 1:100.

b. Enzyme-conjugated SX G-globulin diluted 1:200.

Table 16. Components of media used for isolation of a spiroplasma from X-diseased plants and leafhoppers.

Isolation Medium #1 (CSO medium)

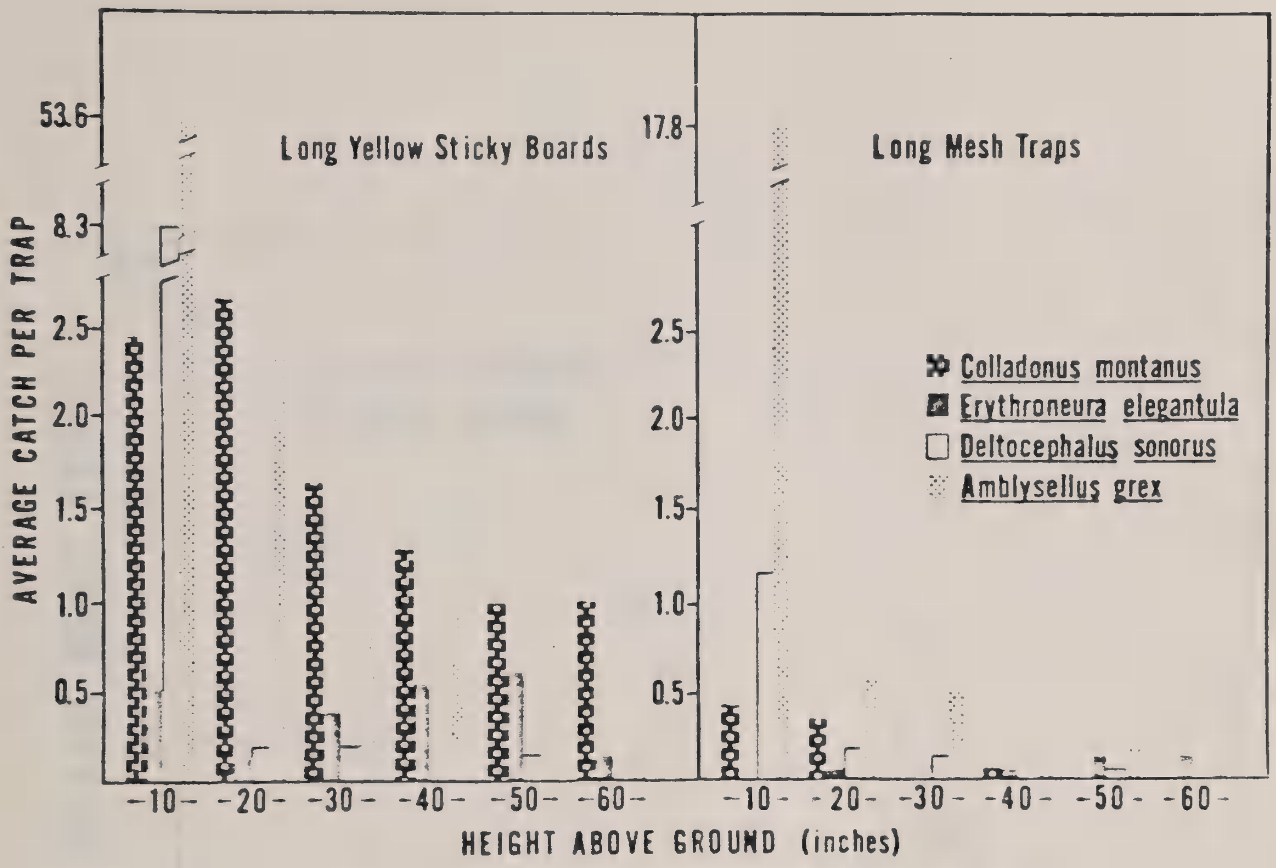
15 g/1.	PPLO Broth Base
160 g/1.	Sucrose
750 ml	OH_2O
.2%	Phenol Red Indicator
10 ml/1.	TC 199
10 ml/1.	CMRL 1066
10 ml/1.	Schneiders Drosophila Medium
150 ml/1.	GG Free heat-inactivators Horse Serum
50 ml	Fresh Yeast Extract

pH = 7.4

Isolation Medium #2 (G/L)

20 g	PPLO Broth Base
50 g	D-Sorbitol
1.0 g	Sucrose
1.0 g	Fructose
1.0 g	Glucose
1.0 g	Tryptone
1.0 g	Peptone
700 ml	OH_2O
200 ml	heat Horse Serum
100 ml	Fresh Yeast Extract

Fig. 2.A.


Long Yellow Sticky Boards
Long Mesh Traps
AVERAGE CATCH PER TRAP
53.6
8.3
2.5
2.0
1.5
1.0
0.5
17.8
2.5
2.0
1.5
1.0
0.5
-10- -20- -30- -40- -50- -60-
-10- -20- -30- -40- -50- -60-
HEIGHT ABOVE GROUND (inches)
Colladonus montanus
Erythroneura elegantula
Deltocephalus sonorus
Amblysellus grex

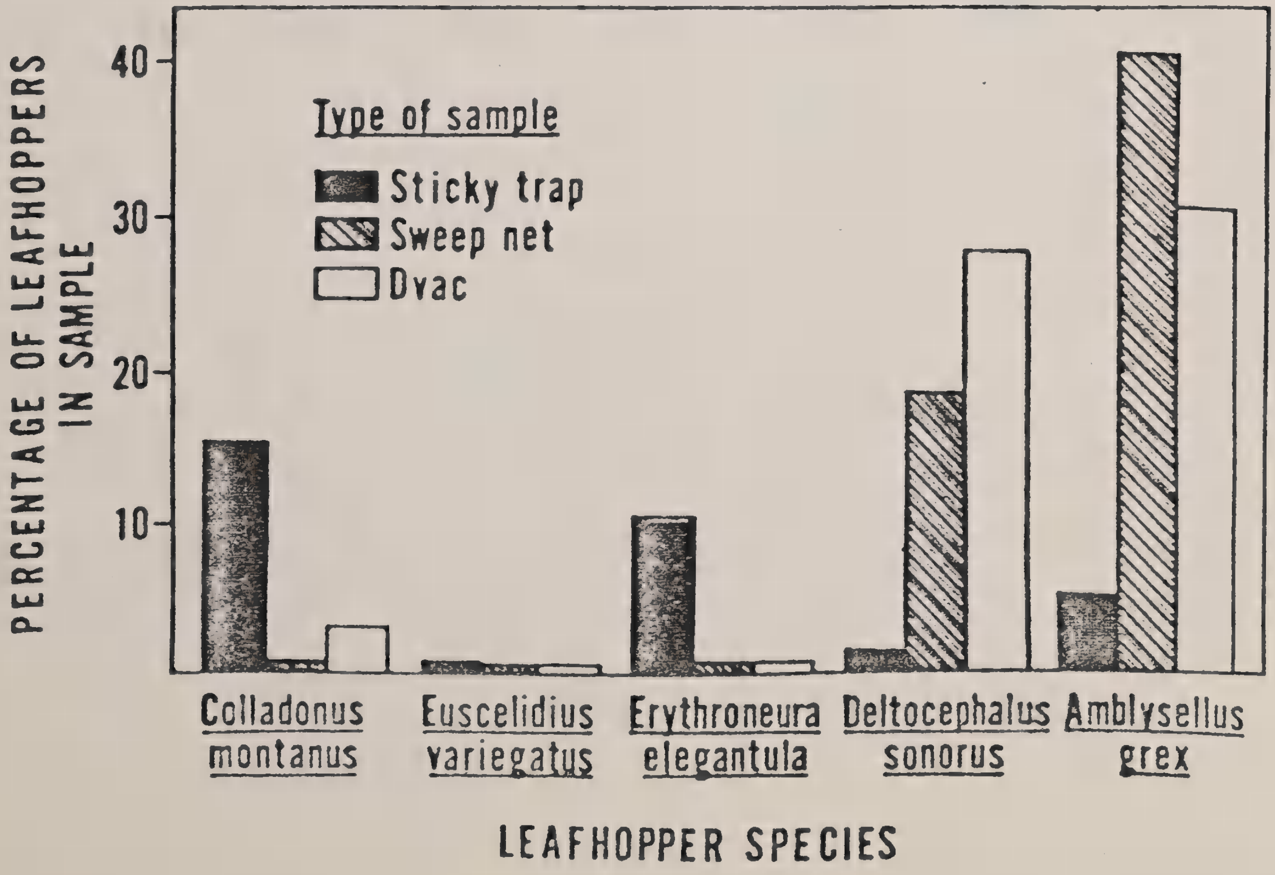
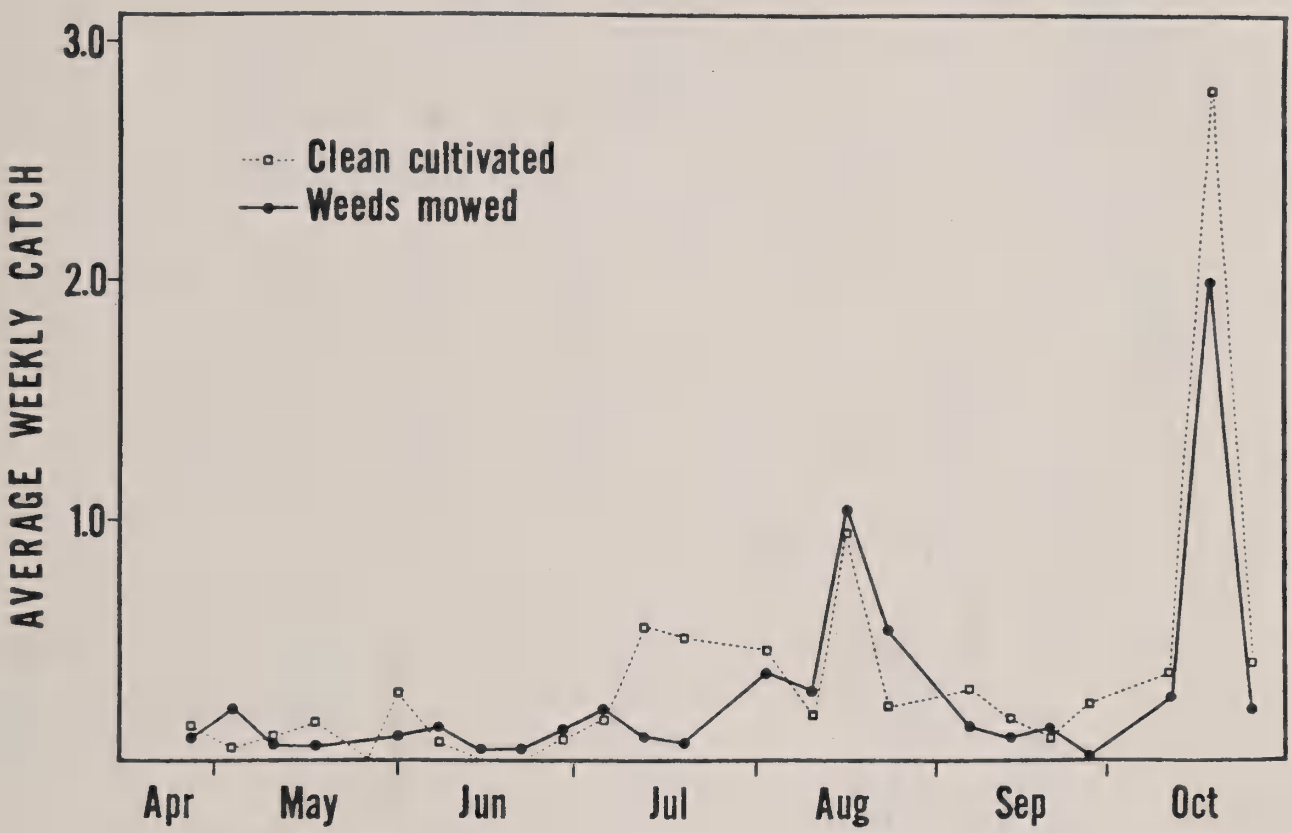

PERCENTAGE OF LEAFHOPPERS IN SAMPLE
40
30
20
10
Type of sample
Sticky trap
Sweep net
Dvac
Colladonus montanus
Euscelidius variegatus
Erythroneura elegantula
Deltocephalus sonorus
Amblysellus grex
LEAFHOPPER SPECIES

Fig. 2. B. Sticky trap catches of *Colladonus montanus* in four clean-cultivated and four sod culture cherry orchards, 1977.



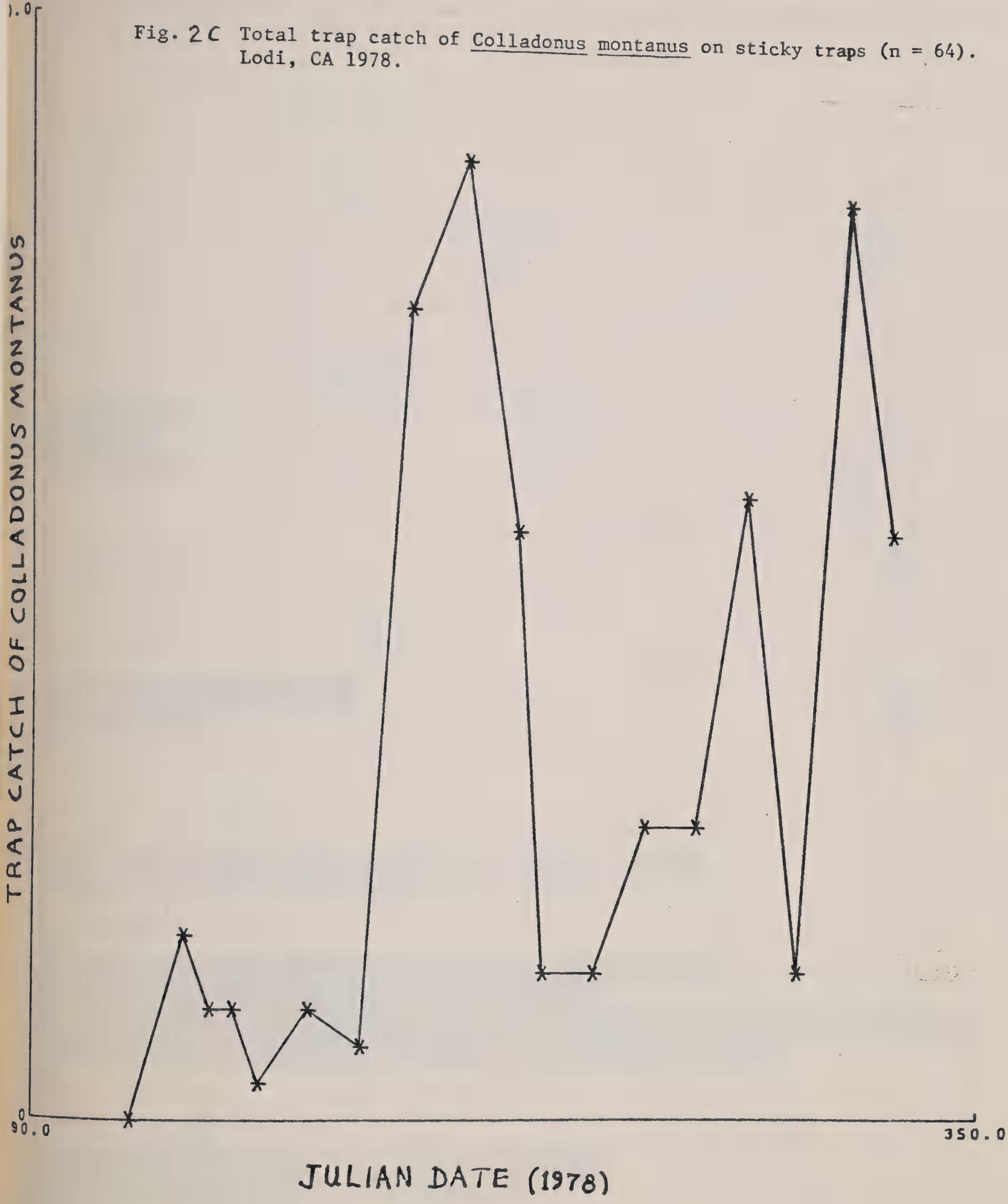


Fig. 2C Total trap catch of <u>Colladonus</u> <u>montanus</u> on sticky traps (n = 64). Lodi, CA 1978.

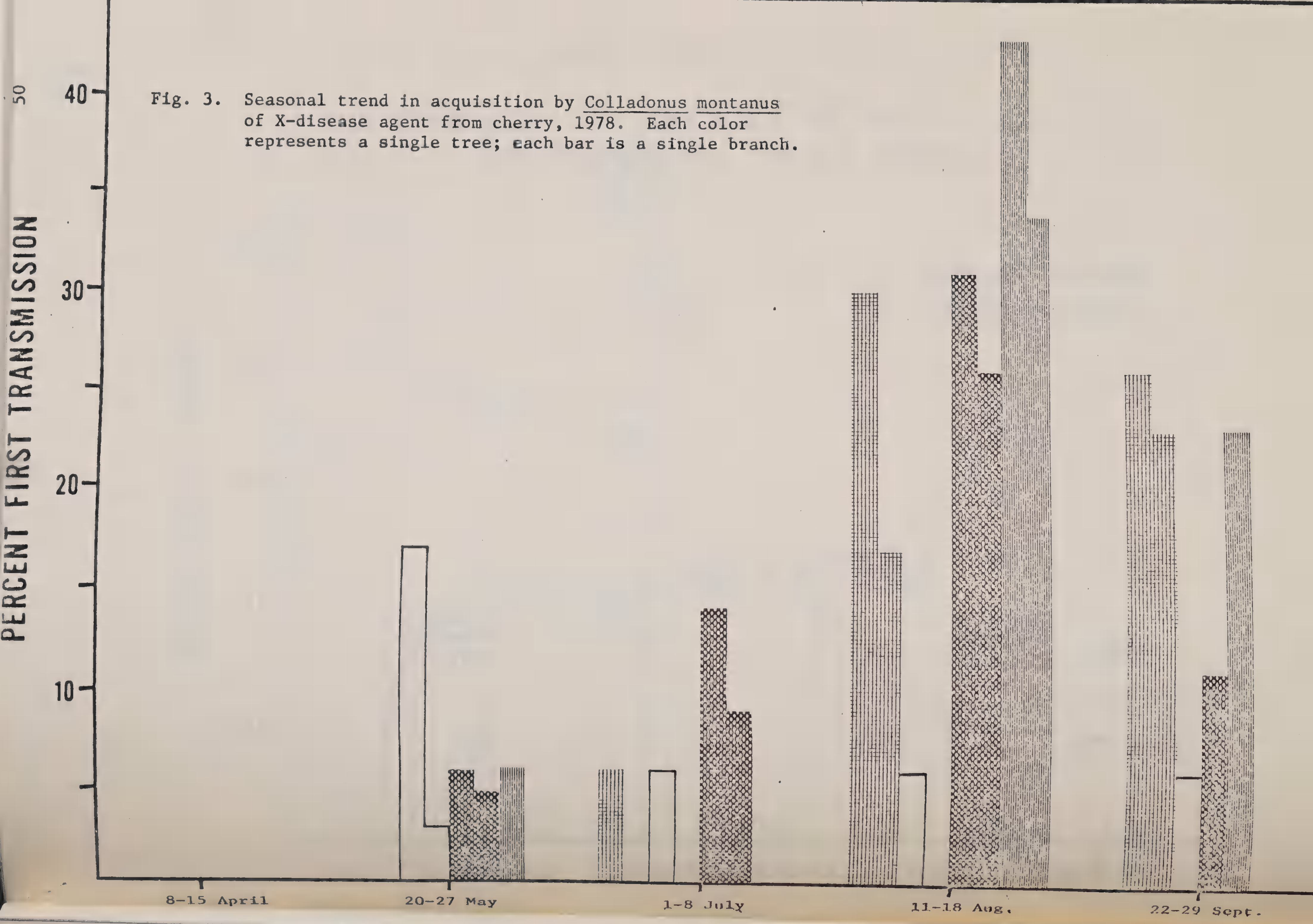


Fig. 3. Seasonal trend in acquisition by <u>Colladonus montanus</u> of X-disease agent from cherry, 1978. Each color represents a single tree; each bar is a single branch.

Fig. 4. Effects of cultivation on leafhopper catches in yellow sticky traps, 1977.

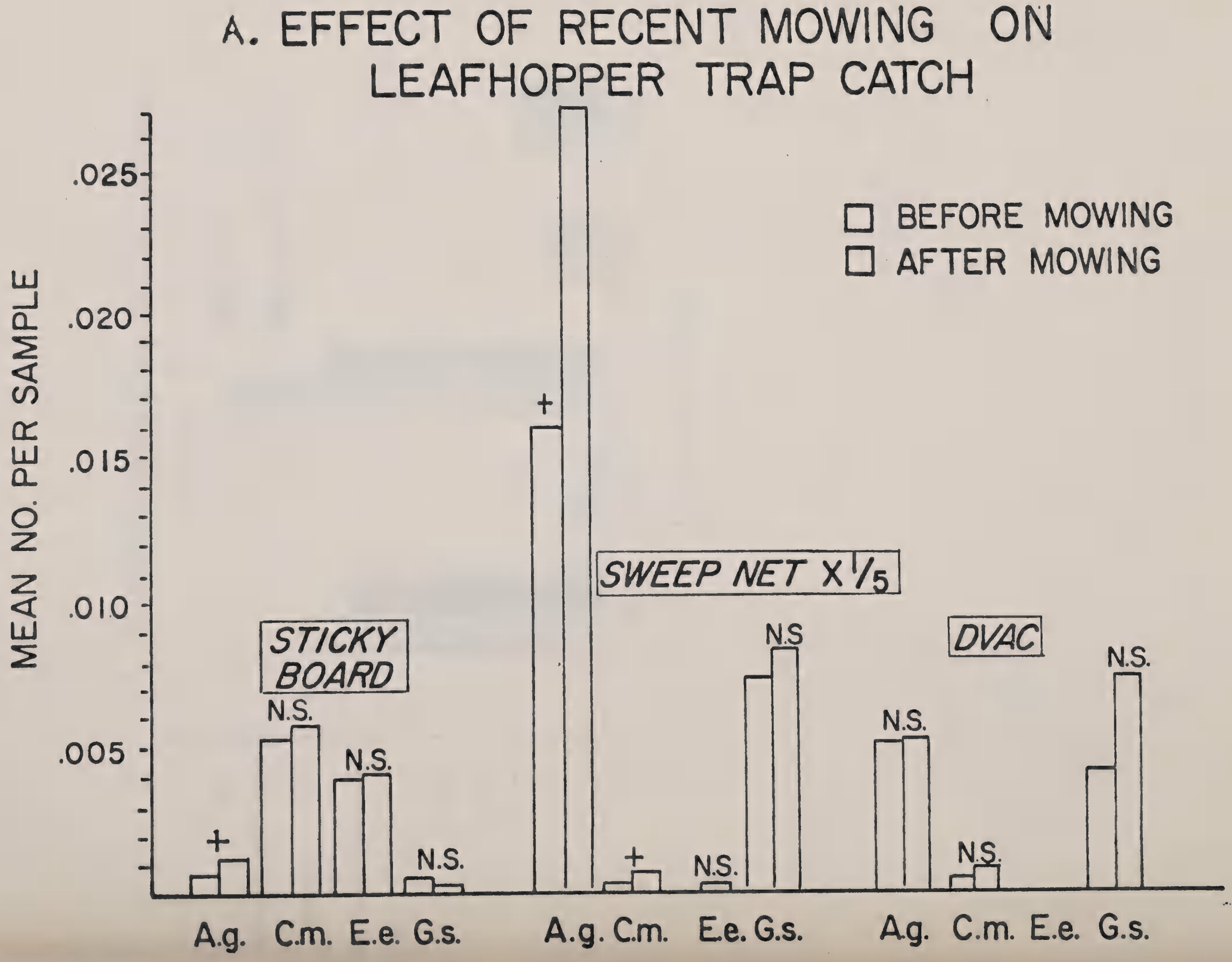


Fig. 4. B. Difference Between Clean and Grass Cultivated Orchards

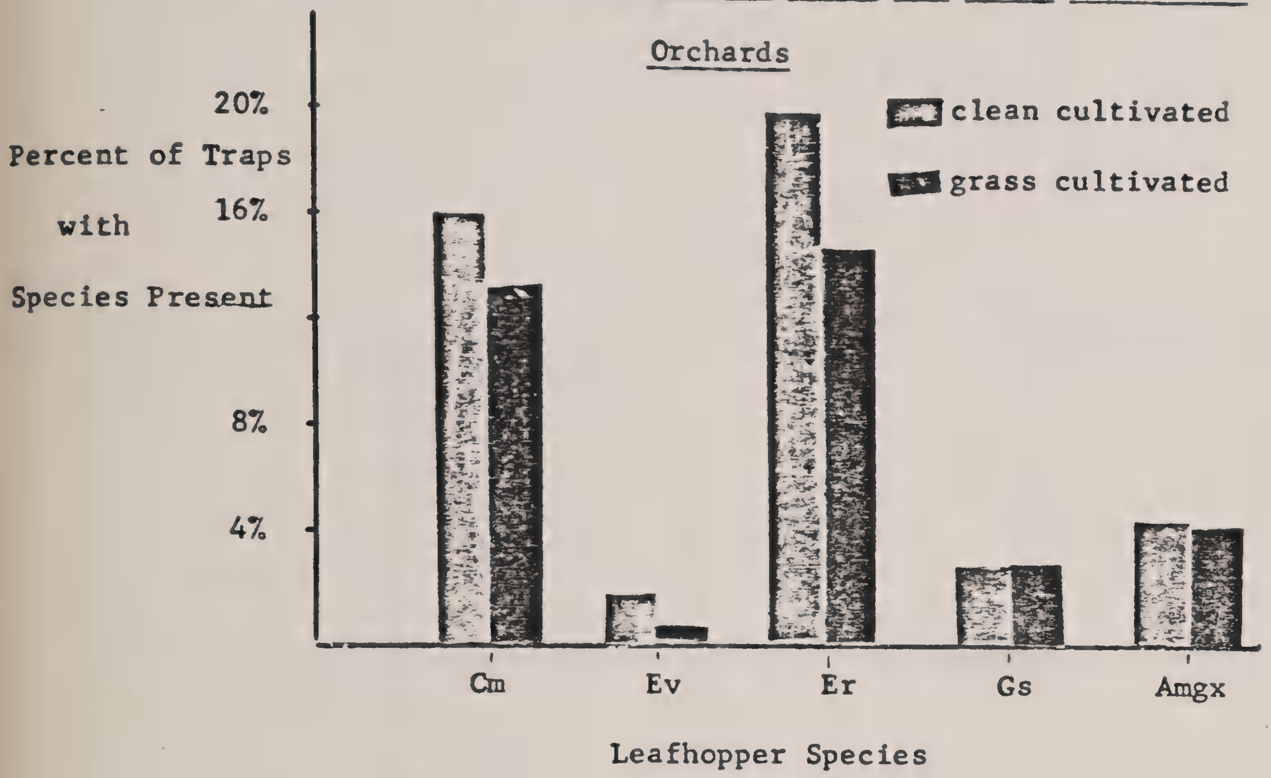
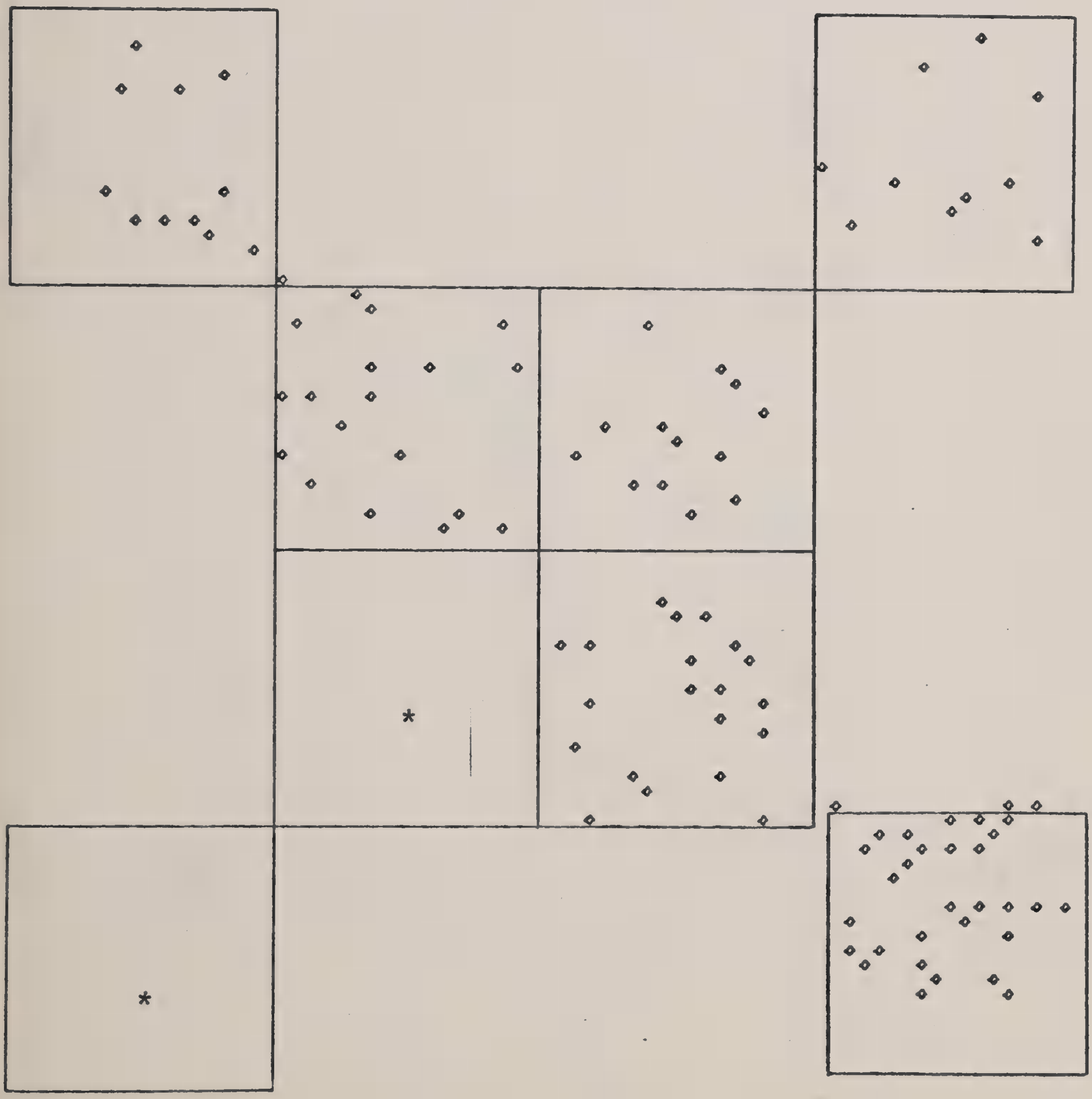


Fig. 5. Distribution of cherry trees with X-disease, 1978, Lodi, CA.

A. Average disease rating below 1.0 (rating scale 0 to 4). The average rating is the average quadrant value for each tree,



* Mapping data intentionally omitted from these two lower left plots because age and rootstocks in these plots differed.

Fig. 5. B. Average disease rating greater or equal to 1.0 and less than 2.5.

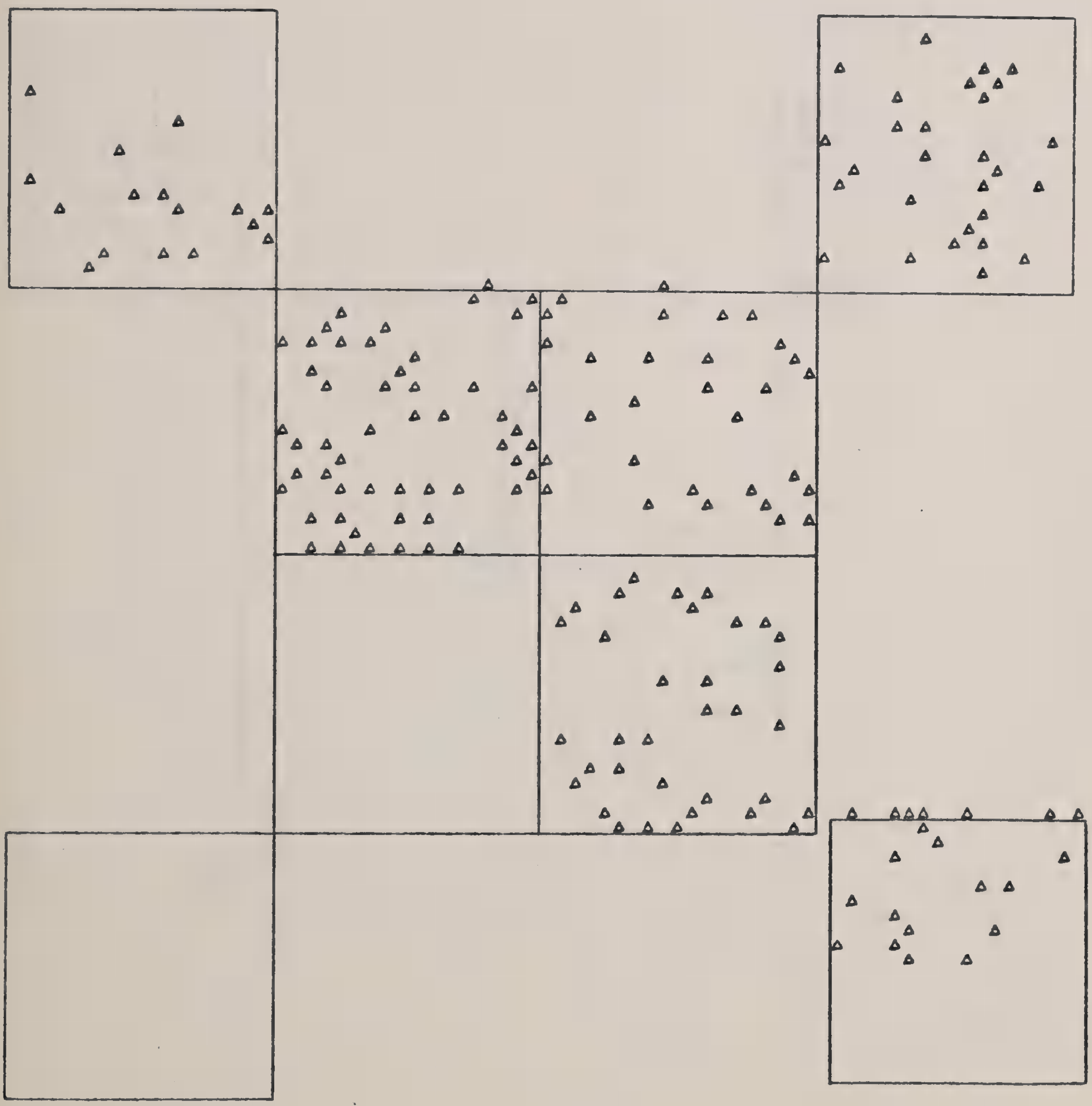


Fig. 5. C. Average disease rating greater than 2.5.

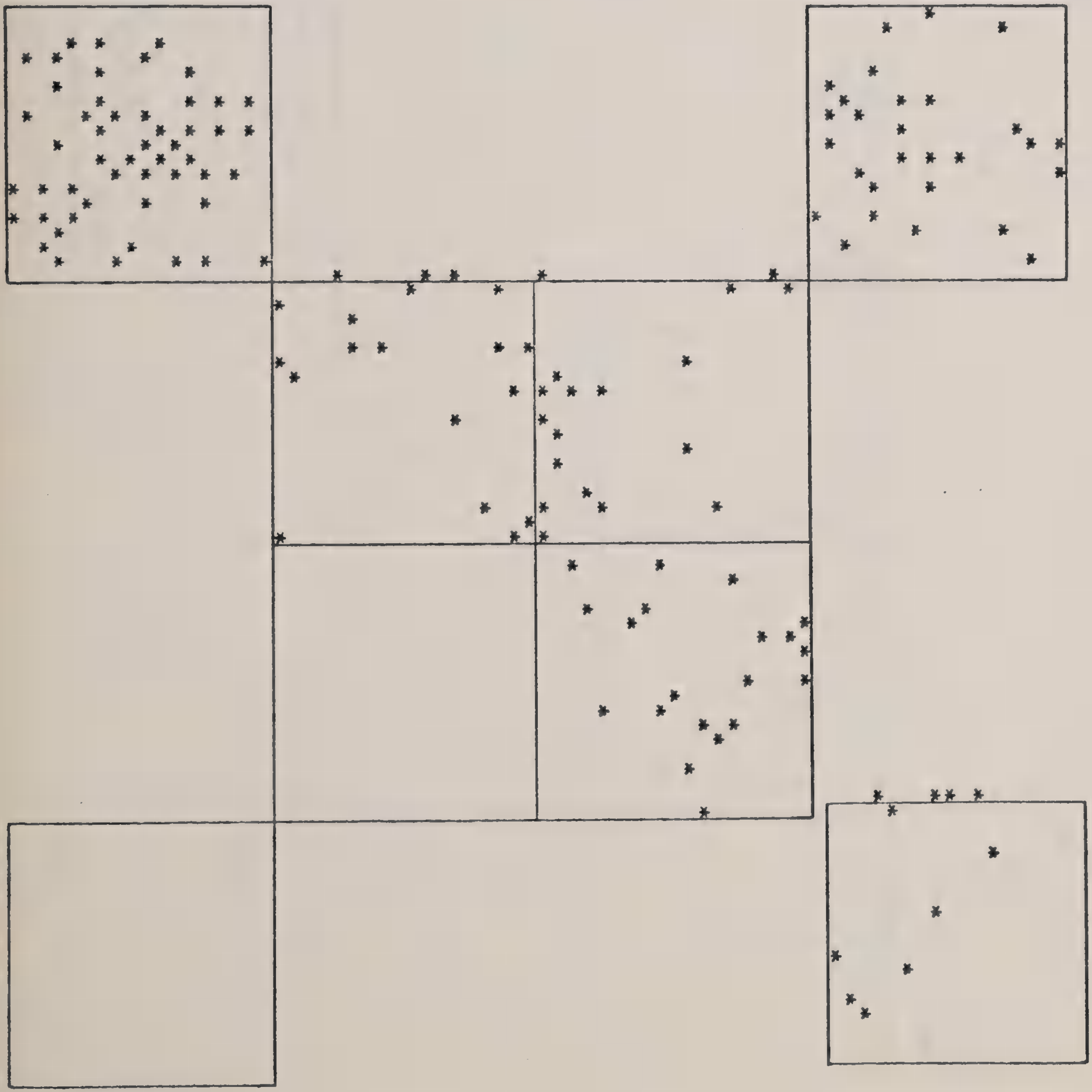


Fig. 5. D. Dead trees.

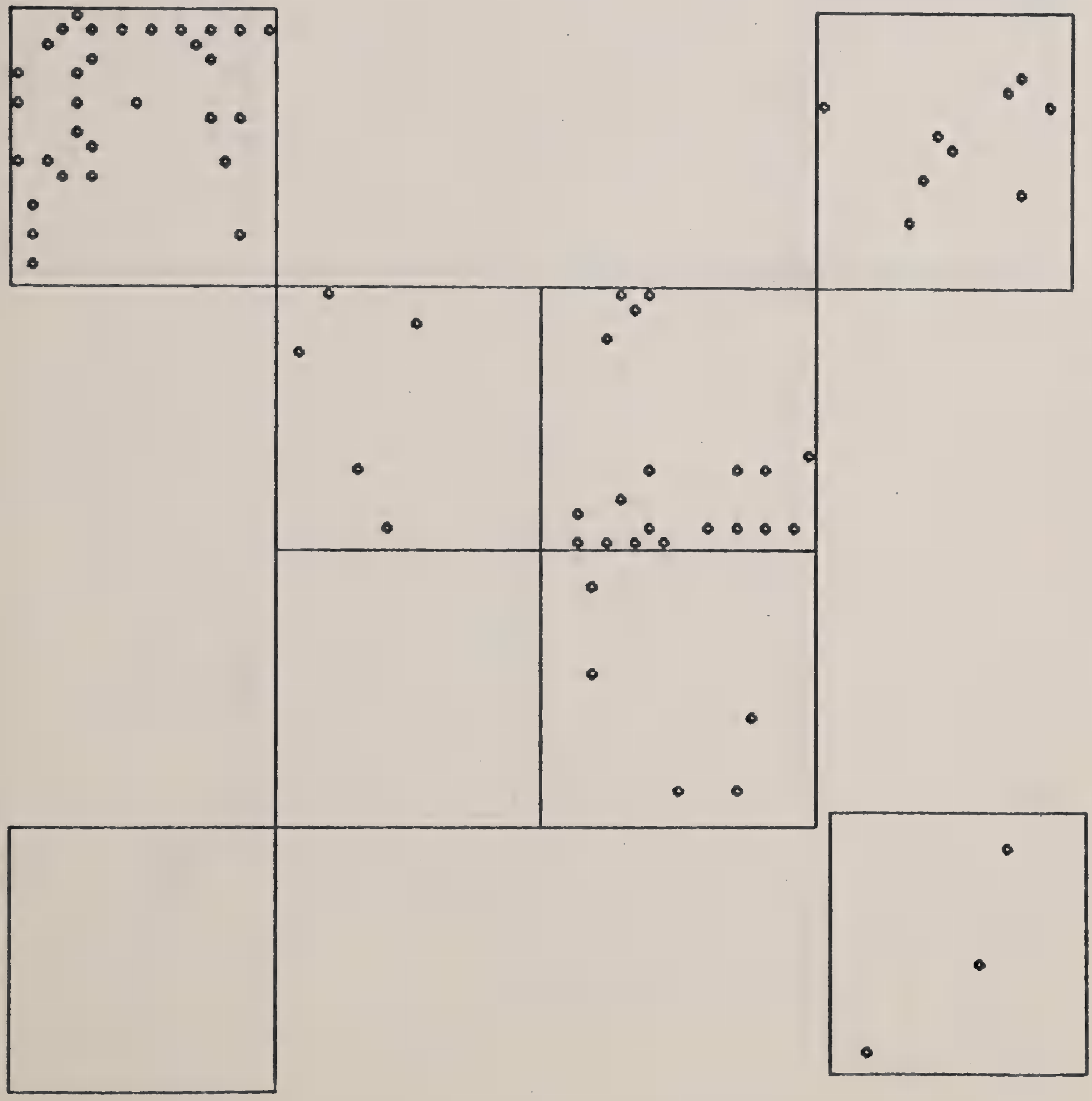


Fig. 5. E. Recently replanted trees.

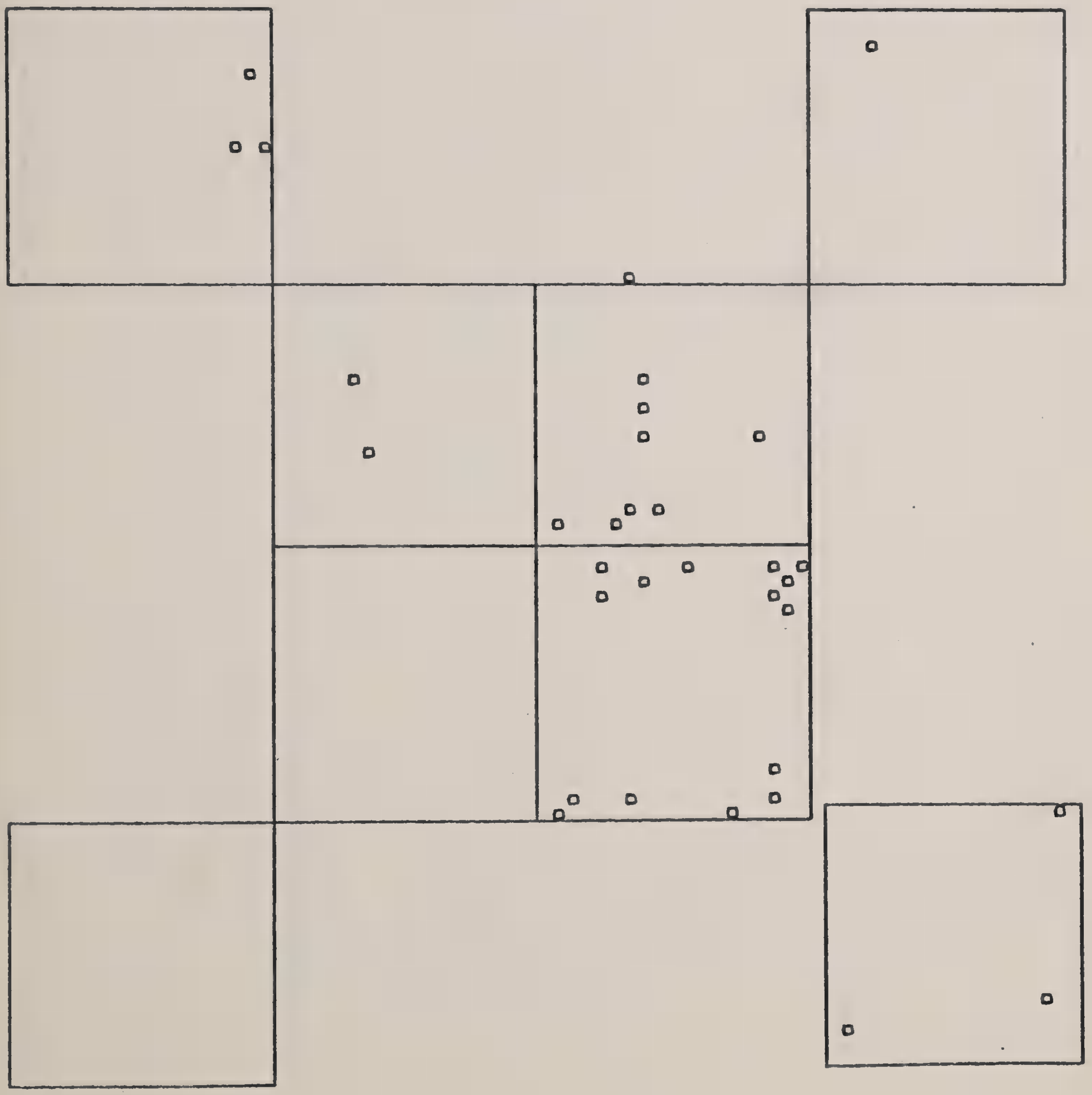


Fig. 5. F. Missing trees.

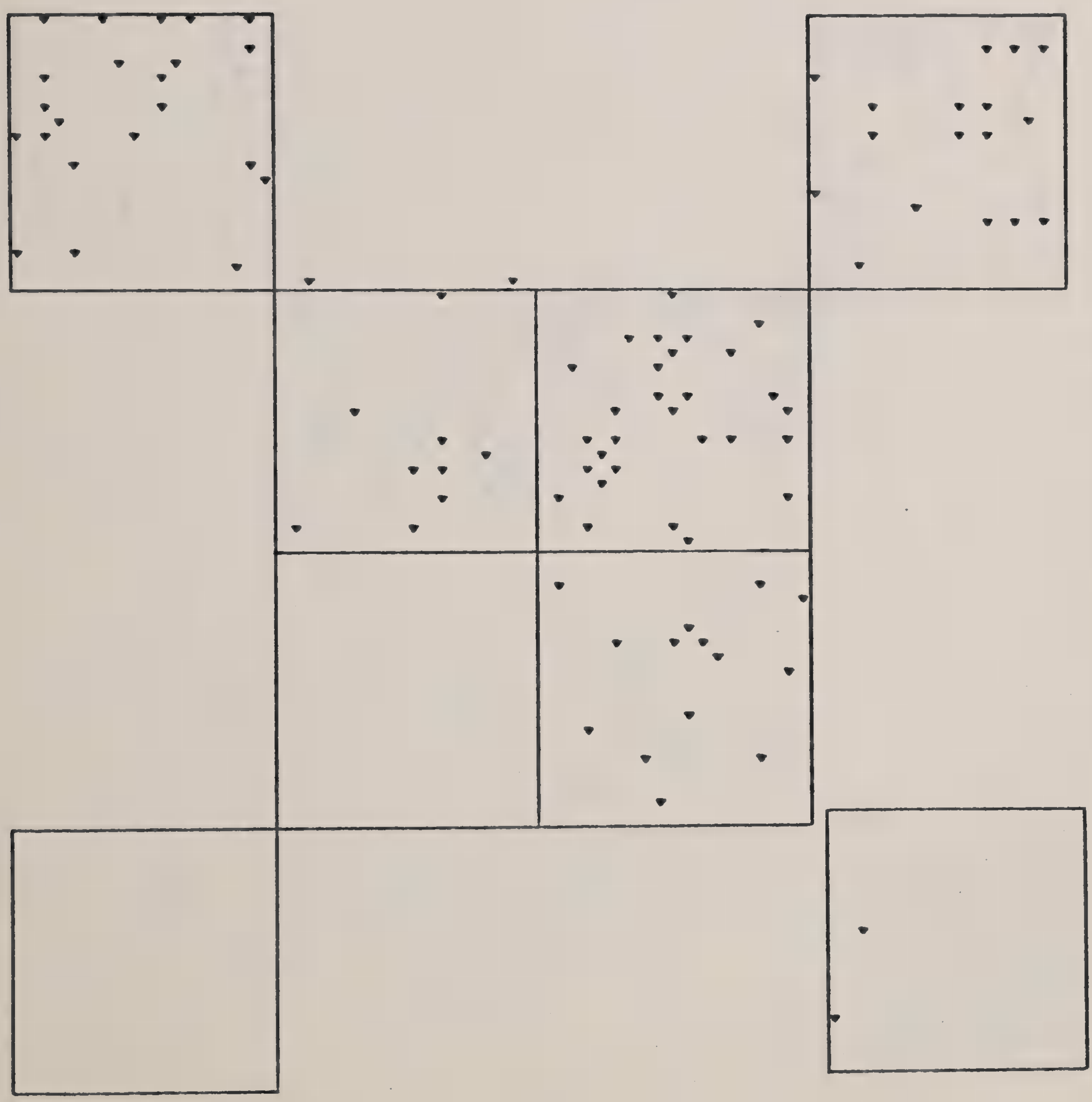


Fig. 5. G. Trees without X-disease symptoms.

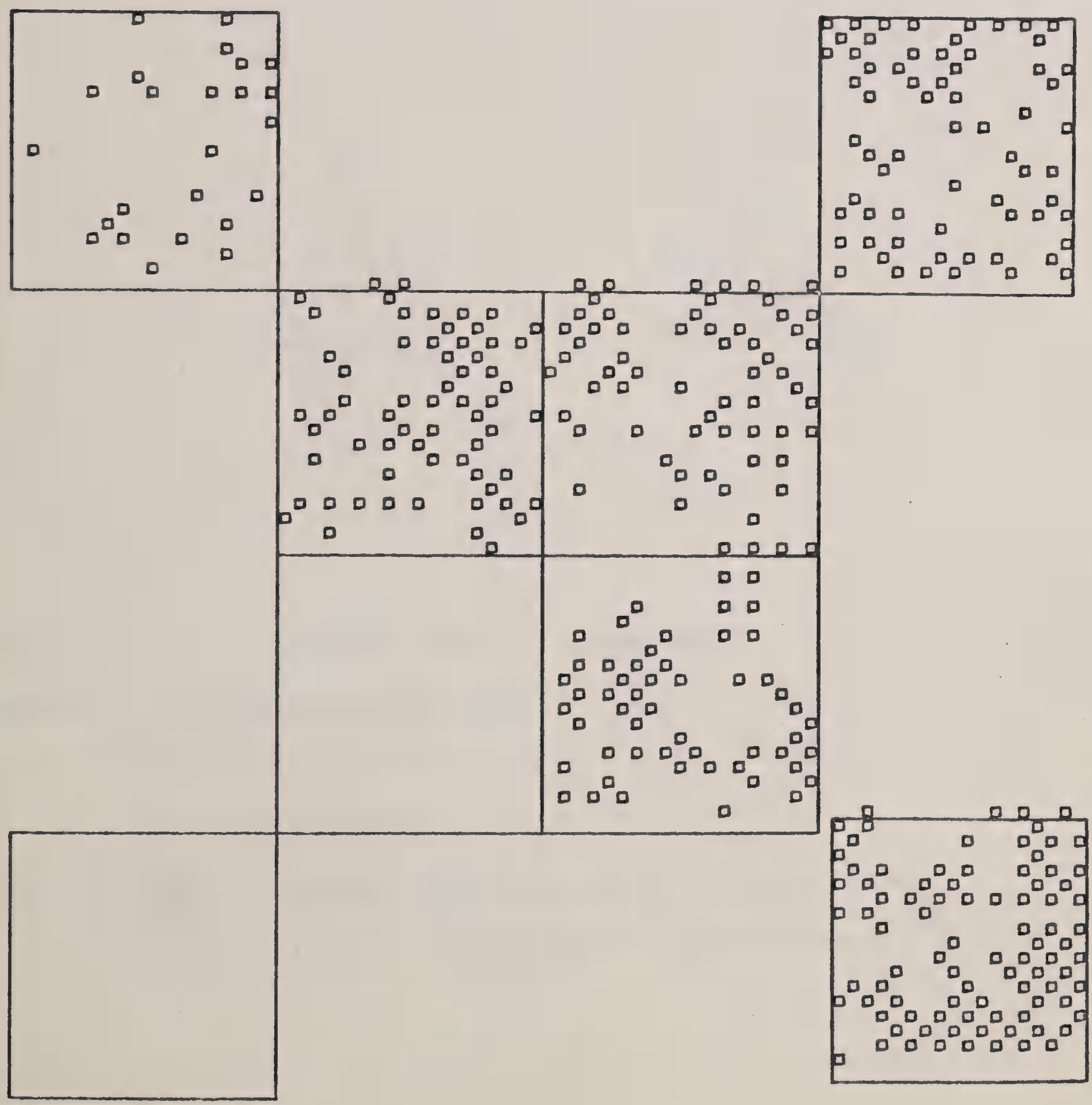
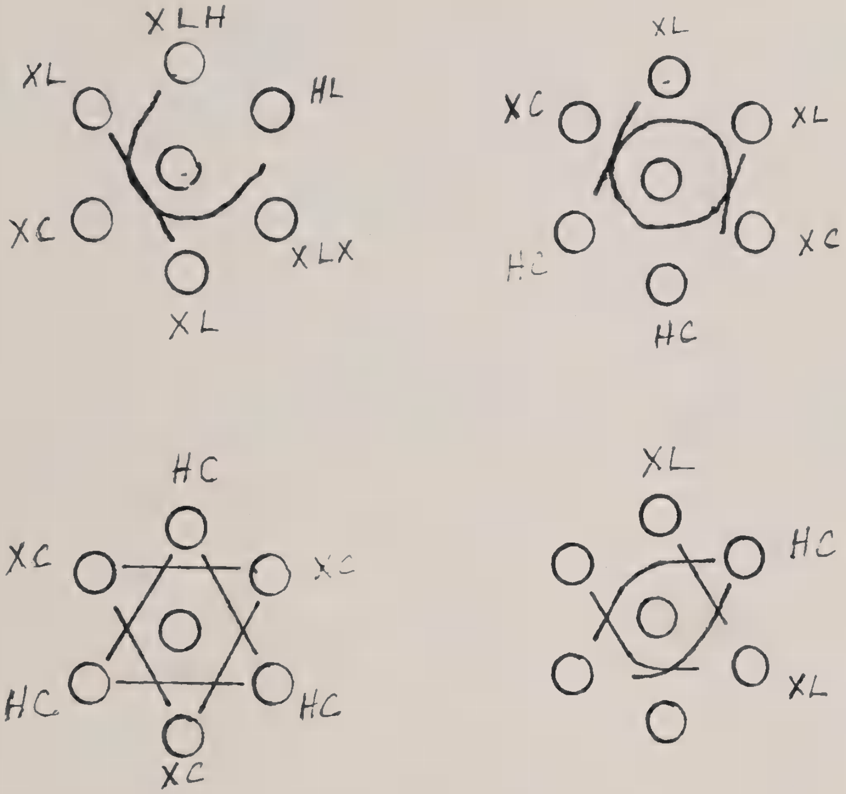


Figure 6. Immunodiffusion patterns of antisera to extracts of diseased and healthy plant and leafhopper materials.



Antiserum: Amt: X-diseased celery in center well

Antigens: XL = X-disease leafhopper

HL = healthy leafhopper

XC = healthy celery

XLX = X-diseased leafhopper fed on X-diseased celery

XLH = X-diseased leafhopper fed on healthy celery

PART TWO

February 1, 1979 - March 31, 1981

INTRODUCTION

X-disease of cherry, locally called cherry buckskin, is caused by the same incitant that causes X-disease of peach. A variant or strain that occurs in California named peach yellow leafroll is lethal to peaches and causes buckskin symptoms in cherry when cherry trees are artificially inoculated.

This project is concerned with the disease in cherry hereafter referred to as buckskin. Previous work under this cooperative agreement was reported under PART ONE, 12-14-5001-272.

The etiology of buckskin is still unknown but mycoplasmalike organisms (MLO) have been consistently associated with the disease and spiroplasma have been isolated from diseased cherry tissues. A few attempts to produce the disease with the spiroplasma isolates were successfull but most were not. Inconsistent isolation of spiroplasma from diseased tissue and similar isolations from tissue of healthy plants have cast doubt on the earlier hypothesis that a spiroplasma was the likely infectious agent causing the disease. Most of these spiroplasma isolates that were injected into the vector leafhopper Colladonus montanus were not transmitted to plants or, if so, caused "atypical" or "mild" X-disease symptoms (9). Only three spiroplasma isolates produced typical X-disease symptoms, but these isolates did not retain pathogenicity after repeated passage in culture media as indicated by the failures of C. montanus to transmit the spiroplasma to celery test plants after these leafhoppers were inoculated with later passages. Based on these results, no clear conclusions could be made as to the pathological role of spiroplasma isolated from X-diseased plants. The true role of MLO or Spiroplasma that are in tissue of diseased trees still needs to be clarified. In the meantime an effective treatment for diseased trees, especially those on Prunus avium rootstocks, has been developed and is available for use by growers. Injection or infusion of Terramycin^R (Pfizer Inc.) into the trunks or scaffolds of affected trees will remit the fruit symptoms of buckskin but longterm results of injection remain to be determined. At present an effective treatment for all affected trees on Prunus mahaleb rootstocks is not available but considerable progress is reported here.

The work reported here was performed cooperatively at Davis and Berkeley. The report is organized into three main sections. Each section has been prepared by the cooperator as indicated.

SECTION I. Dr. S. H. Purcell, Division of Entomology and Parasitology,
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This report summarizes the research accomplished under Cooperative Agreement 12-14-5001-272 (as amended), from February 1, 1979 to March 31, 1981. A previous final report on research under this same cooperative agreement described results from the inception of the project until January 31, 1979. Some results obtained after March, 1981 are also included.

Ecology of leafhopper vectors. In order to establish which insects might be most important in the spread of X-disease, leafhopper sampling methods had to be developed and evaluated. Previous studies (8) of methods of sampling for leafhoppers in cherry orchards concluded that no single sampling method: (1) was optimal for all leafhopper species, (2) measured leafhopper flight or feeding activity equally for all species, or (3) was relatively unaffected by climatic or seasonal conditions. These studies also concluded that, despite some deficiencies, yellow sticky traps were the best overall sampling method for estimating both leafhopper abundance and flight activity. The leafhopper Colladonus montanus was the most abundant known X-disease vector active in cherry orchards and on cherry trees (8). Further studies of C. montanus are reported here. The dispersal behavior and habitat preferences of this insect were primary research objectives. Collections of C. montanus from natural populations in San Joaquin County were also tested for their infectivity with the X-disease agent.

The beet leafhopper Circulifer tenellus is a vector of the spiroplasma plant pathogen Spiroplasma citri (14). As its common name implies, this insect is commonly found in sugar beets grown in low rainfall (less than 20 cm/yr) areas of California. Because the spiroplasmas isolated from X-diseased plants were serologically and culturally similar if not identical to S. citri, the beet leafhopper was evaluated as a possible vector of the X-disease agent and sugar beet as a plant host.

Alternate host plants of X-disease. The susceptibility of several weeds commonly found in cherry orchards or abundant in many sites near cherry orchards was tested as hosts of X-disease isolates recovered from X-diseased cherry from central California. Prior studies had suggested that plant age greatly influenced the susceptibility of some weeds to infection with the X-disease agent, so the relationship of plant age to susceptibility was studied further.

II. LEAFHOPPER TRANSMISSION OF SPIROPLASMA ISOLATED FROM X-DISEASED PLANTS

Details of isolation media and procedures are discussed elsewhere in this report. This section summarizes the methods and results of attempted leafhopper transmission of spiroplasma isolates injected into Colladonus montanus leafhoppers with glass microneedles.

Methods-injections. The leafhoppers Colladonus montanus and Scaphytopius nitridus were colonized on celery (Tall Utah 52-70) in a greenhouse at Berkeley. For transmission experiments, groups of C. montanus nymphs in the 3rd to 4th instar stage were anesthetized with carbon dioxide bubbled through water. The insects were then placed over Parafilm-covered ice to delay their recovery from anesthesia and injected with one of various liquid inocula. The inocula

were prepared by pelleting cultures at 13,000 rpm at 15 min and resuspending the pellet in 10% phosphate-buffered 0.1M sucrose (pH 7.0). The inoculum was examined by darkfield microscopy to determine the morphology and relative density of spiroplasma. Immediately before injection, small drops of the inocula were pipetted onto Parafilm over ice. Glass needles were drawn out from 3 mm tubing by heat on a machine. Each dose was drawn into the needle by capillarity and expelled into the center of each injected leafhopper's abdomen by slight air pressure. The average dose, calculated by weighing, was about 0.02 µl. The injected leafhoppers were transferred to healthy celery plants weekly and insect mortality monitored at each change. All plants exposed to leafhoppers were saved for at least 3-4 months in a heated greenhouse where temperatures ranged from 18 to 40°C, averaging 23-25°C, but this varied with season. After leafhoppers were removed from plants, the plants were sprayed with dimethoate insecticide (Cygon 25WP, American Cyanamide Corp.), recaged for 12-14 days, resprayed with dimethoate and monitored periodically for X-disease symptoms for 3 mo. or longer.

The peach yellow leaf roll strain (PYLR) of X-disease, designated here a PYLRX was maintained in the greenhouse through transmission by <u>C. montanus</u>. This isolate of PYLRX has been maintained continuously in this manner for over 20 years. These PYLRX transmissions served as positive controls to determine when symptoms developed from leafhopper inoculation. Insects not injected or injected with buffered 10% sucrose were negative controls.

<u>Results-Injections</u>. Most spiroplasma isolates injected into <u>C. montanus</u> failed to infect celery as judged by the failure of disease symptoms to develop on test plants or the failure to isolate spiroplasma from the inoculated plants. Three isolates produced typical X-disease symptoms in celery. A few others caused disease symptoms that were not typical of X-disease. Table 1 summarizes these results for 23 spiroplasma isolates from X-diseased plants.

Typical symptoms of PYLRX in celery (6) were produced after 4-5 weeks incubation by three spiroplasma isolates (BL, BM, BP). Transmission usually began two or three weeks after the insects were injected and ceased 3 to 5 weeks after it began (Table 2). Non-infective <u>C. montanus</u> were placed for one week on plants infected with BM and BP isolates ("typical" symptoms). These leafhoppers were transferred weekly and transmitted the PYLRX agent for 3 successive weeks after a 3 week minimum latent period. The plants inoculated from these feeding acquisitions developed typical symptoms of PYLRX in celery, first appearing after an incubation period of 4 to 6 weeks, as is typical of greenhouse transmission at Berkeley. In contrast, the spiroplasma in plants with "atypical" symptoms (BR, BS, TE) were not transmitted by <u>C. montanus</u> or <u>S. nitridus</u> placed on infected plants in groups of 100 for feeding acquisition for one week and then subsequently transferred weekly to healthy celery for at least eight weeks. Disease symptoms were not produced in these test plants and spiroplasmas were not recovered in attempted isolations.

In contrast, isolates BR, BS, and TE produced disease symptoms similar to the first detectable foliar symptoms of PYLRX in celery, but these were noticeable only after a 12 to 14 week incubation period after leafhopper inoculation. Beginning at this time, the oldest outer leaves became slightly chlorotic at the margins of leaflets, usually curling upward slightly. These leaflets were noticeably stiff and brittle. There was no indication of root necrosis. These

symptoms gradually spread over several weeks to other mature leaves, but never to young central leaves. Plants displaying these "atypical symptoms" remained in this condition for over 6 months, in some instances for over 26 months, whereas celery with typical symptoms of PYLRX or other X-disease strains never survive more than 6-15 weeks depending upon strain virulence and environmental temperatures (12). Isolates BR and BS were also inoculated into the leafhopper Scaphytopius nitridus, which subsequently transmitted both isolates, producing the same "atypical" symptoms as the same isolates transmitted by C. montanus. Spiroplasma were readily isolated in vitro from celery plants inoculated with isoaltes BR, BS, and TE, however spiroplasma were not readily isolated from plants inoculated with the three isolates that produced typical X-disease symptoms.

A comparison of symptom severity, incubation period, and insect transmission of spiroplasma isolates (BL, BM, BP) that resulted in typical PYLRX symptoms and of isolates (BR, SB, TE) that produced atypical symptoms is summarized in Table 3.

These results suggested two alternative explanations. First, that a single spiroplasma was isolated from X-diseased plants and that in vitro most of these rapidly lost or altered their pathogenicity to plants, including their insect transmissibility and virulence to infected host plants. A second hypothesis is that the spiroplasmas isolated from X-diseased plants were not the cause of X-disease. For this hypothesis to explain the three isolates that produced typical X-disease symptoms, the hypothesis would have to include the possibility that the actual causal PYLRX agent (spiral or non-spiral) was cultured along with a spiroplasma.

Transmission tests, 1980-81. In 1980-81, eight additional isolates were tested for their transmissibility to celery (Table 4). Two of these injections were from later passages of isolates that produced typical X-disease symptoms. As indicated in Table 4, either the true X-agent or its transmissibility was lost with further passages in vitro. The failure to repeat transmission experiments in which the inoculated plants developed symptoms typical of X-disease casts serious doubts on the hypothesis that the spiroplasmas isolated from plants with X-disease symptoms are the true causal agents of X-disease.

Transmission of Spiroplasma citri. As discussed in other sections of this report [Nyland (III); Kloepper, (II)], spiroplasmas isolated from X-diseased plants were closely similar to S. citri in serological and in vitro cultural characteristics. In order to compare the pathology of S. citri to that of spiroplasmas from X-disease plants, we attempted to transmit S. citri to celery by injecting C. montanus with cultures of this spiroplasma.

A total of eight isolates of S. citri from various localities were injected into C. montanus, S. nitridus, or the beet leafhopper, Circulifer tenellus, using procedures previously discussed. In most cases, the injected leafhoppers were maintained in a 25°C growth chamber under constant light and transferred weekly to fresh plants. In order to prolong its survival, C. tenellus was maintained on sugar beet seedlings and transferred to celery for two or three days, then back to sugar beet.

The results of our attempts to transmit S. citri are shown in Table 5. With four isolates no disease symptoms appeared nor were spiroplasmas reisolated from inoculated plants. These isolates are presumed not to have been transmitted.

Other isolates (Table 5), after 4 to 6 weeks, caused a rapid necrosis of about half to two thirds of some young celery leaflets, which became short, irregular and distorted when mature. Occasionally, after another 4-6 weeks, plants with these symptoms also developed a faint chlorosis and stiffening of leaflets similar to "atypical" symptoms of X-disease. Only a few of the plants exposed to leafhoppers developed any disease symptoms.

Most of the plants that developed leaflet tip necrosis were those fed on by leafhoppers that had been injected with _S. citri_ from two to five weeks before. Decreased transmission to plants after this time might have been due to the smaller numbers of leafhoppers remaining on later transfers and the usual decrease in transmission efficiency of older vector leafhoppers.

Several stalks from _S. citri_ inoculated celery plants were used in attempts to isolate spiroplasmas. The general methods used and detailed results are presented elsewhere in this report. Most isolation attempts were negative, as were attempts to use enzyme-linked immunosorebent assays (ELISA) tests to identify infected plants. There were several exceptions, however. Spiroplasmas were reisolated from plants inoculated (via leafhoppers) with isolates TN. One plant inoculated with isolate TJ gave a positive ELISA test two months after leafhopper inoculation but negative ELISA results four months after inoculation.

An isolate (SC4-2nd passage) from southern California supplied by J. Kloepper, U.C. Berkeley, in April, 1981, was injected into _C. montanus_, _S. nitridus_, and _C. tenellus_ leafhoppers. Each species was transferred weekly to fresh celery plants except that _C. tenellus_ was transferred on alternate weeks between sugar beet and horseradish. Uninoculated leafhoppers served as controls. All test plants were monitored for symptoms for at least 14 weeks. Several plants developed necrosis of the tips of expanding young leaflets about 3-4 weeks after inoculation. ELISA tests of all inoculated plants were negative (Raju, personal communication). One control plant fed on by uninfected leafhoppers was positive for ELISA (Raju). This same plant also had leaflet tip necrosis and spiroplasma serologically identical to _S. citri_ were isolated from it (Kloepper, personal communication). Spiroplasma were isolated from only one other inoculated celery plant--one which never developed any disease symptoms. Horseradish plants inoculated by injected _C. tenellus_ never developed disease symptoms, however, _S. citri_ was isolated on two occasions from one of these plants about 8 months after inoculation. This transmission experiment demonstrated that _S. citri_ could infect both celery and horseradish without producing any distinct disease symptoms after an entire summer of incubation under greenhouse conditions. The infection of a check plant is unexplained. It may have been the result of a labeling error, but suggests that the pathogen-free status of our "non-infectious" leafhopper colonies cannot be trusted absolutely.

A few plants inoculated with _S. citri_ develped symptoms very similar to "atypical" symptoms found in plants inoculated with spiroplasmas that had originally been isolated from X-disease plants. However, in most attempts to transmit _S. citri_ to celery, we did not notice persistent "atyptical" symptoms of diffusely chlorotic, stiff, upwardly curled leaves without any abnormality of young central leaves. Many plants fed on by leafhoppers inoculated with _S. citri_ had necrotic young leaflets 4 to 6 weeks after inoculation exposure to leafhoppers injected with _S. citri_. _S. citri_ was recovered from some but not all of these plants. In summary, it is unclear

whether the differences between the pathological effects of *S. citri* in celery and the effects produced by the "*citri*-like" spiroplasmas originally isolated from X-disease plants are due to inherent differences among the spiroplasma isolates or whether all of these isolates are *S. citri*, but other factors as yet not sufficiently examined might cause differences in symptoms. Temperatures are important in determining what symptoms results from *S. citri* infections (2, 1). This seems to be true in celery as well (Kloepper, personal communication). Our experimental plants were maintained under fluctuating temperatures that varied considerably with the season, which may have confounded the problem of symptom expression. Further research is needed to clarify this question.

III. VECTOR ECOLOGY

Dispersal of Colladonus montanus. Surveys for known or potential leafhopper vectors of the X-disease agent have utilized yellow sticky traps extensively (8, 10). There is, however, no information relating such trap catches to insect movement or to absolute densities, which precludes comparing the abundance of various vector species.

The release and recapture of marked individuals is one method of estimating densities of sparse populations in habitats (trees) that are difficult to sample by more conventional methods or in which accuracy is dependent upon the number of habitat samples (11). We evaluated the practicality of using fluorescent dusts to mark *C. montanus* for orchard dispersal studies. These studies also produced information on the rate and pattern of dispersal of *C. montanus* in an orchard environment.

Rearing and marking. *Colladonus montanus* reared in the insectary at Berkeley were caged on celery for egg-laying for one-week only, and the resulting nymphs were transferred weekly to fresh plants until their release, thus insuring that each colony of leafhoppers was within 7 days of the same age. Colonies of *C. montanus* maintained for many years were used for most experiments except for one (June 1981 release), in which *C. montanus* collected from natural populations near Stockton, CA and colonized for four generations were used.

The day before release, each colony of adult leafhoppers was aspirated using a gentle suction into a 5 cm diameter butyrate cylindrical cage with a foam rubber stopper on one end and plastic mesh at the other end. Fluorescent Radiant^R colored powders (Radiant Color Div., Hercules Inc., Richmond, CA 94702) were dusted through the mesh end of the cage with two puffs from a DeVilbiss Atomizer 15 sprayer (DeVilbiss Co., Somerset, PA 15501). Undusted controls used in some experiments were handled in the same manner except that control insects were not dusted. After dusting, the insects were caged on a large (30 cm) celery plant for transport to the field. The atomizer and cages used in dusting were thoroughly washed after each use to avoid mixing the different colors. The following colors were used: chartreuse (JST-320), cerise ('pink') (JST-9300), deep green (JST-300-321), and orange-red (JST-300-314).

Release and recapture on sticky boards. Releases were made in a 1.1 ha cherry orchard 3 mi north of Vacaville, CA. The cherry trees (cv. 'Bing') were planted squarely 20 ft (6.1 m) apart, as were contiguous prune and almond plants on two sides (Fig. 1). The cherry trees ranged in age from 1 to approximately 20 yr, reflecting the replacement of dead or unhealthy trees over the life of the orchard; however, most trees were older than 10 yr and as a consequence had uniform canopy sizes.

During the preceding 7 mo., this orchard had been surveyed continuously for leafhoppers by keeping eight randomly located yellow sticky boards in cherry trees about 1.4 m above ground. Only three C. montanus were collected during this time.

The central release point was a 1-yr-old tree about 2.2 m tall and 4 cm in diameter at the base. Yellow sticky board traps were coated on both sides with Stikem Special^R (Michel & Pelton Co., Landregan St., Emeryville, CA 94608) (8) and hung from limbs with the trap bottoms at two heights: 0.25 and 1.4 m above the ground. The traps were arranged systematically as shown in Fig. 1. The flat portion of each trap was oriented perpendicular to a line from the release point.

Marked leafhoppers were released shortly after dawn on Oct. 31, 1979, on April 2, 1980, and on June 18, 1981, by removing the cages from the potted celery plants. The celery plants were placed on plastic sheeting to aid in detecting dead insects. On the mornings following release, the plants were shaken to dislodge leafhoppers still remaining. Orchard temperatures were recorded on a spring-powered recording hygrothermometer housed between wooden boxes. The extent of weed growth was surveyed and recorded at release and for several weeks thereafter.

Beginning 15 min after initial release, the control traps were examined for C. montanus. The leafhoppers captured on the boards were gently scraped from the board, placed on labeled index card, and covered with clear plastic film. We carefully removed as much of the leafhopper and surrounding adhesive as possible. We later examined these specimens for the presence of fluorescent dust under 10X to 20X magnification with violet ("black light") illumination. The 16 nearest traps were checked at 15 min. intervals for the first hour, then all traps were examined hourly until dark. On subsequent days the traps were checked just before sunset.

Effects of dusting on survival and flight to traps. To test the possible effects of dusting on longevity and flight activity, 42-day-old C. montanus adults were released in a 4.3 x .9 x .9 m rectangular cage covered with fiberglass mesh and located out of doors at Berkeley. Prior to release 300 leafhoppers were separated into groups of 50 males or 50 females. The 3 groups of each sex were either dusted with fluorescent Radiant Chartreuse, orange-red powder or were not dusted. The leafhoppers were then enclosed on a celery plant and placed inside the larger cage. Four 5" by 4" yellow sticky board traps were hung from the roof of the cage to monitor leafhopper activity. The leafhoppers were released the morning of July 15, 1980; trap catches and mortalities were monitored daily for 2 wk.

To further test the effects of dusting on leafhopper survival, three groups of 65 adult leafhoppers each were dusted with Radiant Chartreuse powder, then each group was placed into a cage that contained a large celery plant. The same number of undusted leafhoppers were placed on three celery plants as a control. Daily counts of survivors were made.

RESULTS

Effects of fluorescent dusting on survival and trap captures. Mortality of two groups of 100 dusted leafhoppers over a one-week period was not greater than that of 100 undusted controls from the same colony. The dead bodies of 25 "chartreuse", and 19 "orange-red", and 25 undusted controls were collected from the bottom of 3 respective cages in this period. mortality after one week was not measured because ants scavenged the dead leafhoppers. In a second experiment, three replicates of *C. montanus* dusted "orange"red" survived longer than undusted controls. For dusted insects, the weekly average survival was 71%, 65%, and 52% for three wk after dusting. The survival of undusted controls was 59%, 59%, and 11%.

Dusting had no effect on the sticky trap catches of *C. montanus* released into a large cage (t-test, 14 df, $p>0.33$ for "orange-red"; $p>0.86$ for "chartreuse").

Reliability of marking with dusts. Samples of leafhoppers dusted with one of four different colors or left undusted wre coded by one person and identified by another. Of 22 insects, only one was misidentified. In a second test using 36 insects, no errors were made.

Special patterns of trap catches. In all three field release experiments, trap catches were highest on traps nearest the release point and decreased exponentially with distance as shown in Figs. 1 and 2. Equations from eight different dispersal models (Taylor, 1978) were fitted to the data. The best fit was achieved with the equation: $N = \exp(a + bx^{-1})$ where N is number of insects at distance x; a is the intercept, and b the slope of the linear regression equation. This is a special form of Taylor's (1978) general model: $N = \exp(a + bc^{c})$ where $c = -1$. For the October release this gave a correlation coefficient (r) of 0.934 (d.f. = 7) and 0.752 for the April release. Captures were highest to the north and east for both the October (Fig. 1) and April (Fig. 2) releases. Half of the insects (0.75% of 2950) captured after the third release in June, 1981, were from southerly traps, although winds were generally from the west and northwest on all release dates.

Traps placed 0.25 m above ground caught only a few more *C. montanus* that did traps 1.4 m above ground. The lower traps caught 51.6% of the total after the October release and 59% after the April release. There were no significant differences in captures on the sticky trap side which faced the release point ("front") or away from it ("back"). "Front" sides capture 58% in October and 48% in April.

Time of capture. Most leafhoppers remained on the celery plants after the cage was removed. Only a few made short flights of a few feet immediately after release. As shown in Tables 6 and 7, there were significant differences among release dates as to when most released insects were captured. After the late October release, many *C. montanus* were trapped after 3-10 days following their release (Table 6). In contrast, after the April release, 82% of captures were made within the first 2 days (Table 7).

After the June, 1981 release of 2960 C. montanus, 17 were trapped the first day, 5 the following two days, and none the next four days. This was a very low percentage recovered (0.75%) compared to the two other field releases. The June release was to test for differences between C. montanus colonized in the laboratory for many years and leafhoppers colonized only 3-4 generations from field collections. Since recently colonized C. montanus represented 13 of the 22 insects trapped, there was no evidence that the length of colonization affected trap capture results.

The dispersal behavior of C. montanus on different release dates probably was greatly influenced by temperature and weed cover. Temperatures were relatively cool following the release in October, with an average maximum of 21°C and minimum just above freezing. Weed cover was extensive and lush. After the release in April, the daily maximum temperature was 30°C and minimum was 5.5°C. Moreover, the orchard had just been cultivated, leaving only a few dried grasses and other weeds around tree trunks. In June, 1981, maximum temperatures averaged 38°C and minimums were 19°C. Again as in April, 1980, weed cover was negligible. trap catches 1 wk after the November and April releases suggested that after several days the released leafhoppers were generally dispersed throughout the orchard.

Sex ratio of captures. When C. montanus were released into a large cage, the ratio of males:females on the sticky traps was 1:1 and 2:1 for the first 2 days, then dropped to 1:2 for the next 2 days, and after 10 days was 1:9. In catches following the April release (Table 7) predominately males were trapped during the first few days, but this trend reversed sharply thereafter. After the June release, the male:female ratio was 2.8 and 1.5, but these data were based on low numbers. The dispersal behavior of young female C. montanus may differ considerably from the relatively old adults used in these experiments. The sex of leafhoppers recovered form the autumn release was not determined.

CONCLUSIONS--Mark and Release Studies

1. Dusting with fluorescent powder is a reliable method of marking C. montanus. There were no effects on longevity or dispersal of dusted leafhoppers; however, accurate identification of markings requires close examination, which limits the usefulness of this method for large recapture experiments.

2. The spatial pattern of trap captures of C. montanus released from the central release point fits reasonably well to dispersal models in which density decreases exponentially with distance from the initial dispersal point.

3. Trap recapture rates varied greatly on different release dates, possibly because of the effects of orchard temperature and weed cover on the dispersal of C. montanus. Thus, yellow sticky trap catches would be difficult to convert to accurate population indices.

4. Mark-recapture methods using fluorescent dusts would provide estimates of absolute populations where natural populations of C. montanus are large enough so that a reasonable number of marked insects would be recaptured. Approximately 1 to 20% of released leafhoppers can be expected to be recaptured on sticky traps. Natural populations of C. montanus are too sparse under most orchard conditions to utilize the recapture method of estimating populations.

Biology of C. montanus in sugar beet fields. In the spring of 1980, our attention was brought to sugar beet fields as a possible source of X-disease vectors. A mature cherry orchard south of Linden, California, that was rapidly collapsing with X-disease and had been located next to sugar beet fields in recent years, was surveyed with sticky traps and by sweep net. Above-average (8) numbers of C. montanus were collected. During the autumn of 1980, this and several other beet fields were monitored for C. montanus using sweep net (15 inch) samples of 50 sweeps per sample.

The average numbers of C. montanus in these samples in 1981 are illustrated in Fig. 8. Several bean fields were also monitored at the same time, but without finding C. montanus.

The relative abundance of C. montanus, as determined by sweep samples, was low and variable throughout the summer months. Two or three insecticide applications per field were normal for all fields from late June through August. Populations of C. montanus apparently were greatest in beet fields during late autumn and late spring months (Fig. 3). We noticed no significant differences in sweep net catches of C. montanus in weedy portions of beet fields or in relatively weed-free fields or portions of beet fields. Nymphs were very rare in sweep samples from beet fields. Thus, it is not clear whether or not beet plants are significant as breeding hosts of C. montanus.

We compared the survival and reproduction of C. montanus on beet in the insectary at Berkeley. A total of 50 adult C. montanus were caged separately on beet and celery plants for eight days. On celery, 36% of the leafhoppers survived, whereas none were alive on beet after eight days. Nymphs were present on both plants two weeks later, but about 30% fewer nymphs were present on beet then celery. A second repetition of this experiment produced almost exactly the same results after one week: 34% survival on celery and none survived on beet. In transmission tests described later in this report, C. montanus survived longer than 1 week on beet but still died sooner than on celery. Further studies on nymphal development on beet are needed, but these results demonstrate that C. montanus can survive for short periods and can reproduce on beet in the laboratory.

In San Joaquin County, sugar beets usually are not harvested until the spring after planting. Because C. montanus is relatively abundant in beet fields during autumn and spring months, we investigated: (i) the dispersal of C. montanus to nearby cherry orchards following beet harvest in May 1981, (ii) the transmission of the X-disease agent by natural populations of this leafhopper, and (iii) the possibility that sugar beet is an alternative host of the X-disease agent.

Dispersal of C. montanus to cherry after beet harvest. The first two of these three aspects of X-disease epidemiology were studied by placing 16 sticky traps systematically in each of 5 cherry orchards near beet fields just before beet harvest. These traps were changed periodically for over a month thereafter. Sticky traps were maintained in cherry trees at a height of 1.4 m as previously described (8), except that yellow 0.5 mm thick plastic vinyl boards rather than painted wooden boards were used. The traps were placed in each orchard in four separate transects of four traps each. Each transect was perpendicular to the cherry-beet boundary. Traps were placed at 9, 33, 58 and 83 m from beet fields. At least 6 sweep net samples of 50 sweeps per sample were made in beets before harvest and in cherry orchard weeds before and after the beet harvest.

After harvest, one orchard adjacent to a beet field with an average of over 3 *C. montanus* per 50 sweeps experienced a sharp increase in trap catches for three weeks after harvest. There was no clear gradient of leafhopper catches relative to distance from beet in this orchard (Fig. 9) or in the other 4 orchards, in which *C. montanus* catches were low. However, these results (Fig. 4) suggest that beet harvesting did greatly increase *C. montanus* populations in cherry.

Natural infectivity of C. montanus with X-disease agent. Sweep net collections of *C. montanus* were made in beet fields and adjacent cherry orchards surveyed from September 1980 until November 1981. The live insects collected were caged on celery at Berkeley and transferred to fresh celery plants weekly or biweekly until all insects had died. Despite the normal survival of these insects on celery and the fact that celery is a relatively sensitive indicator host for X-disease, no transmissions of the X-disease agent to celery were noted for a total of 503 *C. montanus* collected over 17 different dates or sites. We can conclude that natural infectivity of *C. montanus* in and near cherry orchards is low (99% confidence interval of 0.01 to 0.04).

Transmission tests--beet leafhopper (Circulifer tenellus). The beet leafhopper, *Circulifer tenellus* is often found in California sugar beet fields in low rainfall (less than 20 cm/yr) areas of central and southern California. This insect is a vector of *Spiroplasma citri*. In order to further investigate the possibility that sugar beet fields might have a role in the epidemiology of X-disease in cherry orchards, we evaluated *C. tenellus* as a possible X-disease vector.

Methods

Two methods were used to inoculate *C. tenellus* leafhoppers: (i) natural feedings on X-diseased plants and (ii) injecting infectious extracts of the X-disease agent. Infectious extracts were prepared by grinding the detached heads (5 µl phosphate-buffered 10% sucrose per head) of *C. montanus* leafhopper that had been fed on X-diseased celery for more than 30 days. Late (3rd or 4th) instar nymphs were injected as described for *C. montanus*. Injected *C. tenellus* were maintained on sugar beet (US 742) for 2-3 weeks and then transferred to celery, peach, or periwinkle (*Vinca rosea*) for 2 or 3 days. Because *C. tenellus* survived poorly on non-beet plants, they were alternated between beet and either celery, periwinkle, or peach, spending 2 or 3 days on non-beet plants. Uninjected leafhopper controls were transferred in the same manner. All plants exposed to *C. tenellus* were sprayed with dimethoate and monitored for disease symptoms for 3 months or longer.

Results--Circulifer tenellus transmission trials. *C. tenellus* that were injected with PYLRX extracts transmitted the PYLRX agent to celery, periwinkle, and peach, causing typical X-disease symptoms in these plants. Moreover, some of the sugar beet plants that had been fed upon by these leafhoppers developed disease symptoms associated with X-disease: severe stunting, root necrosis, and marginal leaf chlorosis followed by necrosis of leaf tissue. Diene's staining (4) of root and leaf tissues from beets with these symptoms were strongly positive for beet roots but not for leaf veins. Phloem elements in the roots of afflicted beet plants stained dark blue with Diene's stain, which is a positive test for mollicute infection of plant tissues (3). Plants exposed to control leafhoppers did not develop disease symptoms. Attempts to isolate spiroplasma from symptomatic or healthy peach, periwinkle, celery, or beet were

unsuccessful. PYLRX-injected *C. montanus* that transmitted PYLRX agent to celery and periwinkle were also exposed to sugar beet plants, with similar results. *C. montanus* injected with GVX extracts also transmitted this agent to celery and apparently to beet, as judged by the appearance of disease symptoms in the beet plants.

We evaluated the ability of *C. tenellus* to acquire the PYLRX agent by feeding on infected celery and on what we presumed were PYLRX-infected beet plants. Non-infectious *C. tenellus* nymphs were placed on PYLRX-celery or beet plants for a total of at least 7 days. The leafhoppers were caged on celery plants for only 2 or 3 days then alternated to beets for 2 days and back to PYLRX-celery. No transmissions to celery were noted for *C. tenellus* treated in this manner in two experiments begun with over 100 *C. tenellus*. No transmission was noted for *C. montanus* leafhoppers that were exposed for 3 days or longer on beets with X-disease symptoms (from both GVX and PYLRX strains) and subsequently transferred to celery for 10 weeks.

Conclusions--Beet leafhopper transmission of X-agent. The beet leafhopper appears to be an experimental vector of the PYLRX and GVX causal agents. Transmission was relatively efficient by *C. tenellus* that had been inoculated with infectious extracts. However, there was no evidence that the beet leafhopper can transmit the X-agent after feeding on X-disease plants.

Sugar beet appears to be a pathological host of PYLRX and GVX agents, as judged by the appearance of disease symptoms and a positive Diene's test in inoculated beets that had symptoms similar to the symptoms of X-disease in a variety of plants (7), but does not appear to be a source of inoculation for further leafhopper spread. This latter point should be tested further with large leafhopper populations, because even relatively inefficient transmission of X-agent from sugar beet might be significant to the spread of X-disease to perennial crops such as cherry and peach.

IV. WEEDS AS HOSTS OF X-DISEASE AGENTS

The X-disease agent has a rather wide range of host plants (7), especially considering that relatively few species have been evaluated as possible hosts. We found (5) that some weeds commonly found in cherry orchards seemed to be susceptible as young seedlings to infection with the X-agent but older plants were quite resistant to infection. We attempted to expand on these preliminary observations because the possible involvement of weeds as sources of inoculum of the X-agent is a little studied but potentially important part of the epidemiology of X-disease.

The plants selected to be tested as potential hosts of the XDA are common weeds in cherry orchards in San Jose County, CA: mustard (*Brassica campestris*), filaree (*Erodium circutatum*), and fiddleneck (*Amsinkia intermedia*). *Colladonus montanus* was the vector used in these studies because it is the leafhopper vector of the XDA most abundant in California cherry orchards.

METHODS:

After a 35-day latent period, naturally fed GV-infective C. montanus were caged on filaree, mustard and fiddleneck of varying ages for inoculative purposes (Table 8). Infective leafhoppers either singly, or in groups of 3, 5 or 10 depending on the plant size to be inoculated, were given a one-week inoculation access period on the weeds. After this period, the insects were removed and the plants placed in the greenhouse for symptoms development.

Non-infectious leafhoppers, in groups of 20, were placed on the infected weeds exhibiting XD symptoms for reacquisition purposes. After 1 week, these leafhoppers were removed and placed on celery, and transferred weekly.

RESULTS:

Transmission of the XDA to mustard, fiddleneck and filaree by C. montanus following feeding on diseased source plants revealed that younger plants are markedly more susceptible to infection than are older plants. Reacquisition attempts were made by caging healthy C. montanus on hosts exhibiting symptoms of XD. Reacquisition occurred 4-6 weeks later only from fiddleneck inoculated at 14 days of age.

The common orchard weeds, filaree, fiddleneck and mustard, are susceptible to infection with X-disease with C. montanus. Susceptibility to infection in these weeds species decreased rapidly with plant age. Reacquisition from infected weed plants generally was inefficient.

V. LEAFHOPPER TRANSMISSION OF GVX-AGENT FROM CHERRY TO CHERRY.

In 1980, spiroplasmas were isolated from cherry trees with GVX symptoms. We wanted to transmit the GVX agent from these trees to healthy cherry to see when symptoms would appear in cherry following leafhopper inoculation to trees on either 'Mazzard' or on 'Mahaleb' rootstocks. Further, we wished to evaluate these trees for recoverability of spiroplasma.

Methods. In early July, 1980, non-infectious C. montanus late instar nymphs were caged for one week on two X-diseased cherry trees on mazzard rootstocks in a San Joaquin Co. orchard. The leafhoppers were returned to Berkeley and placed on celery for 2 weeks so that they would complete their latent period. After this, the leafhoppers were caged on one of four cherry trees in the experimental farm of the Department of Plant Pathology at U.C. Davis on September 2, 1980. Two 'Lambert' cherry trees were on mahaleb rootstocks and the other two on mazzard roots. All trees were low-grafted and several years old. An equal number of adjacent trees (two on each rootstock) served as checks. The trees were made available by S.M. Mircetich from an experimental plot established for root and crown rot research. Although some of the trees in the portion of the experimental block had died or were declining from undetermined causes, all of the inoculated and check trees we used were apparently healthy. Two separate groups of 14 presumably GVX-infective C. montanus were caged on teminals of branches each of the four inoculated trees.

Two weeks later the cages were examined. No C. montanus remained alive. The branch and cages were sprayed with insecticide and two weeks later, the cage was removed and the branch tagged. We monitored these trees during 1981 for X-disease symptoms. On several dates in 1981, non-infectious C. montanus nymphs reared at Berkeley were caged on these trees in attempts to recover GVX-agent. The leafhoppers used in these trials were caged for one week on the inoculated trees and then tested for subsequent GVX transmission by transfers to celery. At various times, foliage and fruit samples were used in attempts to isolate spiroplasmas from the inoculated and control trees.

Results--symptoms. The first X-disease symptoms appeared in early June in trees grafted on Mahaleb rootstocks. At this time, inoculated trees on Mazzard roots were identical in appearance to controls. On May 21, leaf symptoms were a slight chlorosis and curling or twisting of mature basal leaves. These leaves developed a faint reddish tinge on their underside. By July, all leaves drooped noticeably. Infrared thermometry (Raynger II, Raytek Corp.) measured a leaf temperature on June 12, 1981 of 90° and 91°F in full sun on inoculated 'Mahaleb' trees and 86°-88° on the inoculated 'mazzard' trees and other uninoculated trees. Shaded leaves were all 78°F on all symptomless trees and 80° and 81° on the symptomatic trees. By July 14, both trees on Mahaleb roots were wilting noticeably. The leaves on these trees dried up completely during August.

Inoculated trees on Mazzard roots did not have disease symptoms until late August, at which time one tree developed slightly chlorotic basal leaves and reduced shoot growth. The other trees had some faintly chlorotic leaves compared to the untreated checks.

The GVX-agent was transmitted by C. montanus that had fed on inoculated trees, but only from trees on Mazzard rootstocks. Leafhoppers caged on June 18-24, 1981, all died, probably because of hot weather. Leafhoppers caged on the tree on Mazzard roots that first developed disease symptoms transmitted GVX to celery at Berkeley. The leafhoppers caged on trees on Mahaleb rootstocks survived well on these trees for a week but failed to transmit from them, despite the more advanced state of decline of the trees on Mahaleb roots.

Attempts to isolate spiroplasma on DG-2 medium were made by J. Kloepper and D. Garrott at Berkeley from leaves and fruit collected on June 18, 1981. All attempts failed to produce evidence of microbial growth, as judged by a color change in the neutral pH indicator in the DG-2 medium or by turbity (D. Garrott, personal communication).

Conclusions. Although only two trees of each rootstock type were inoculated, C. montanus readily transmitted GVX from cherry to cherry, and the resulting progression of X-disease symptoms in the inoculated "Mahaleb-rootstock" trees was very similar. It is not yet certain that both trees on Mazzard roots were infected.

Late season inoculations of cherry on Mahaleb rootstocks produced tree collapse in July. Trees that collapse in June presumably are inoculated earlier than September of the previous year. Trees on Mazzard apparently do not produce fruit symptoms during the spring of the year following late season (after August) infection.

LIST OF TABLES

Table 1. Transmission to celery of spiroplasma isolates injected into *Colladonus montanus* leafhoppers, Berkeley, 1979.

Isolates	Original source	At time of injection:			
		passage level	time cloned	dilution	symptoms
1. RA	peach (field)	8	2x	10^{13}	none
2. RE	peach (greenhouse)	5	1x	$2x10^{8}$	none
3. RG	cherry	10	0	$9x10^{15}$	none
4. RN	celery	2	0	$2x10^{2}$	none
5. RX	peach	3	0	$4x10^{2}$	none
6. BJ	peach	3	0	$4x10^{2}$	none
7. BK	celery	4	1	$8x10^{6}$	none
8. BL	celery	3	0	$8x10^{5}$	typical
9. BM	*Colladonus montanus*	3	0	$8x10^{5}$	typical
10. BN	peach	4	1x	$8x10^{6}$	none
11. BO	celery	4	0	$8x10^{6}$	none
12. BP	peach	5	0	$8x10^{7}$	typical
13. BR	peach	6	1x	$8x10^{9}$	atypical
14. BS	celery	5	1x	$8x10^{7}$	atypical
15. BT	celery	5	1x	$9x10^{7}$	none
16. BU	*C. montanus*	5	2x	$9x10^{7}$	none
17. BV	celery	6	3x	$9x10^{9}$	none
18. BW	celery	4	0	$9x10^{5}$	none
19. TA	celery	3	0	$4x10^{2}$	none
20. TB	peach	5	2x	$9x10^{7}$	none
21. TC	cherry	4	0	$9x10^{5}$	none
22. TD	cherry	4	0	$9x10^{5}$	none
23. TE	celery (BR)	4	0	$9x10^{5}$	atypical

Table 2. Transmission from *in vitro* cultures that produced typical X-disease in celery, 1979.[a]

isolate	date injected	no. leafhoppers	weekly tranfers infected	total[b] transfers
BL	6-15	112	3,4,5	7
BM	6-29	100	2,3,4	8
BP	6-19	100	3,4,5,6,7	9
check (no injection)	6-15	50	---	12

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a. *Colladonus montanus* injected intrahaemocoelically with concentrated inocula.

b. Total number of weekly transfers with 5 or more leafhoppers still alive. Later transfers with less than this number were made in most experiments.

Table 3. Comparison of characteristic pathogenicity and transmissibility of isolates that induced "typical" or "atypical" X-disease symptoms in celery.

Isolates	"typical" (BL, BM, BP)	"atypical" (BR, BS, TE)
Characteristics		
1. Symptoms (23-28°C)		
a. 4 weeks	mottled chlorosis, stiff leaves, roots stunted and brown	none
b. 5-6 weeks	necrosis of small roots, faint yellowing of new growth, severe stunting	none
c. 7-11 weeks	severe chlorosis, almost complete root necrosis, most plants wilt and die	none
d. 12-15 weeks	wilt and die or already dead	mottled chlorosis, stiff leaves, rootlets slightly necrotic or appear unaffected
e. 15-24 weeks	dead	as above; some plants have darker green leaves with marginal chlorosis or necrosis
2. Insect transmission		
a. by injection	transmitted by *Collodonus montanus* (Cm), *Scaphytopius nitridus* (Sn) after a 2-3 week latent period	transmitted by Cm, Sn after a 2-3 week latent period
b. after feeding on symptomatic plant	readily transmitted by Cm	not at all (or poorly) by Cm, Sn
3. Isolation of spiroplasma from tissues of symptomatic plants	occasional at best (5%)	consistantly (90%)

Table 4. Tests of Colladonus montanus transmission to celery of spiroplasmas isolated from X-diseased plants, 1980-81.

Isolate	Source of Isolate	Transmission Results	reisolation[a/]	Elisa[b/]
TC, TD	GVX cherry	no transmission	not done	not done
TE, UE	GVX cherry	"atypical" symptoms in 6th, 7th transfers	+	+
UF	GVX peach	no transmission	not done	not done
TA	PYLRX celery	no transmission	not done	not done
UJ	"atypical" celery (TH)	no transmission	-	-
TB	PYLRX peach	no transmission	-	-
TE-CK, TB-CK UE-CK	(check)	no transmission	-	-

a/+ = spiroplasmas reisolated only fromg symptomatic plants
* no spiroplasmas reisolated from symptomatic plants only

b/+ = Optical density reading over 4 times uninfected controls from symptomatic plants only.

Table 5. Tests of leafhopper transmission of *Spiroplasma citri* to celery

Isolate	Source[a]	Leafhopper[b] injected	*S. citri*[c] reisolated	ELISA[d] tests	Symptoms
1. TI	Southern California	*Colladonus montanus*	no	negative	none
2. TJ	Southern California	*C. montanus*	no	one weak positive	one plant with leaflet tip necrosis
3. TK	Southern California	*C. montanus*	no	negative	one plant with tip necrosis
4. TL	Arizona	*C. montanus*	no	negative	Three plants with stiff older leaves
5. TN	Southern California	*C. montanus*	no	negative	none
		S. nitridus	yes	one week	two plants with leaf tip necrosis; followed by stiff, chlorotic older leaves
6. TO (TQ,TX)	Southern California	*Circulifer tenellus*	no	N/A	none (leafhoppers alternated between celery & beets)
		C. montanus	no	negative	One plant tip necrosis of young leaves
7. TW	Southern California	*C. montanus*	no	negative	none
8. TY	Illinois	*C. tenellus*	N/A	N/A	one plant--stiff chlorotic leaves

Table 6. Yellow sticky board captures of marked *Colladonus montanus*, October 30-December 10, 1979

	Percent captured on						
Color/age[a/]	day 1	day 2	day 3	days 4-8	days 9-10	days 11-15	total 1-41
Y/92	5.6	1.4	0.9	6.0	0.9	0	15.3
P/78	1.2	0	0.5	0.3	0.5	0.5	4.2
G/71	0	0	0	1.8	0.2	0.4	2.5
R/57	b/	b/	0	0.9	0.9	1.8	4.3
none/50	b/	b/	0.1	4.1	4.0	4.8	16.0

a/ Pigment colors (and numbers released): Y = "chartreuse" (215), P = "cerise" (403), G = "deep green" (512), R = "orange-red" (650), none = not dusted (779). Age in days after oviposition.

b/ "R" and "none" released on 3rd day of experiment.

Table 7. Yellow sticky board captures of marked <u>Colladonus</u> <u>montanus</u>, April 1 - 25, 1980

	Percent captured on						
	day	day	day	days	days	days	total
Color/age[a/]	1	2	3	4-7	8-13	14-21	1-25
Y/92	5.6	1.4	0.9	6.0	0.9	0	15.3
R/99	9.9	3.4	1.3	0.9	1.7	0.9	18.1
(: ratio)	(14.5)	(14.0)	(6.5)	(1.0)	(1.3)	(1.8)	(8.0)

[a/] Color of dusts (and numbers released): Y = "chartreuse" (900), R = "red-orange" (232).

Table 8. Transmission of GVX from infective <u>Colladonus</u> <u>montanus</u> to several weed hosts

Weed tested	plant age (days) at time of inoc.	number of leafhoppers/ plant	fraction transmitting (& percentage)	estimated % of single insect transmission
Fiddleneck	7	1	0/10 (0)	0
(<u>Amsinckia</u>	8	1	9/10 (90)	90
<u>intermedia</u>)	14	1	0/10 (0)	0
	23	5	0/10 (0)	0
	57	10	0/3 (0)	0
Filaree	2	1	12/25 (48)	48
(<u>Erodium</u>	3	1	3/10 (30	30
<u>circutatum</u>)	7	1	6/10 (60)	60
	9	1	8/10 (80)	80
	12	3	11/35 (31)	10.3
	14	3	8/35 (23)	7.7
	19	5	5/10 (50)	10
	20	5	4/10 (40)	8
	28	5	0/25 (0)	0
	60-90	10	0/25 (0)	0
	2-3	1	15/35 (43)	43
	7-9	1	14/20 (70)	70
	12-14	3	19/70 (27)	9
	19-20	5	9/20 (45)	9
	28	5	0/25 (0)	0
	60-90	10	0/25 (0)	0
<u>mustard</u>	8	1	8/10 (80)	80
	14	3	0/10 (0)	0
	22	3	0/10 (0)	0

LIST OF FIGURES

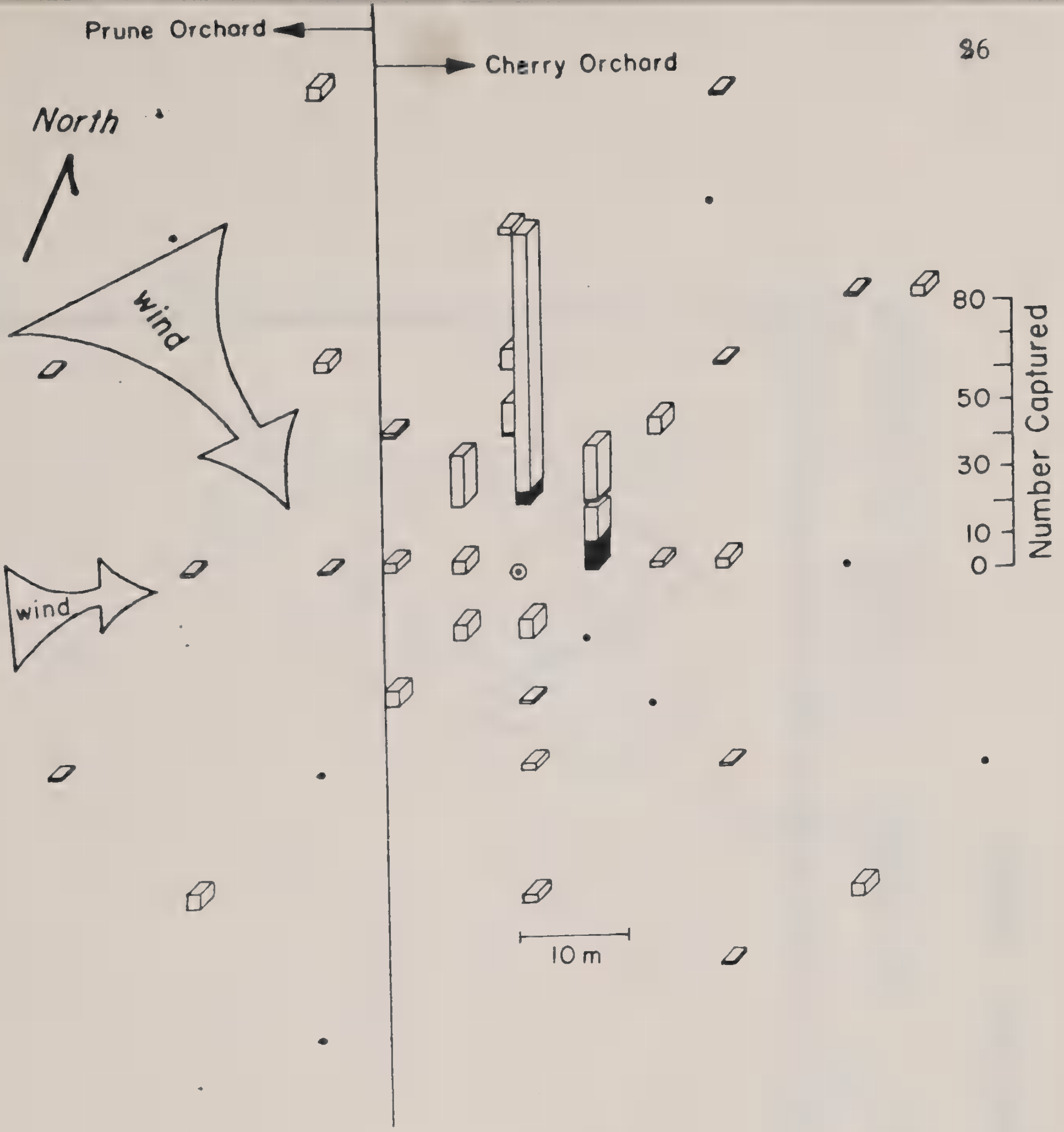
1. Sticky trap catches of Colladonus montanus released from a central point in a cherry orchard and adjacent prune orchard on October 31, 1979. Darkened bases of columns indicate the first day's catch. Arrows indicate general direction of prevailing winds for most of the first week.

2. Sticky trap catches of Colladonus montanus released from the same point as in figure 1 but on April 1, 1980, darkened bases of columns indicate the first day's catch. Arrows show the prevailing winds for the first three days after release.

3. Average numbr of Colladonus montanus swept from beet fields, San Joaquin, Co., 1981.

4. Average sticky trap captures of Colladonus montanus (A) before and one week after beet harvest in an adjacent field and (B) in the next 4 weeks after beet harvest.

g. 1


Prune Orchard
Cherry Orchard
North
wind
wind
80
50
30
10
0
Number Captured
10 m

[illegible]

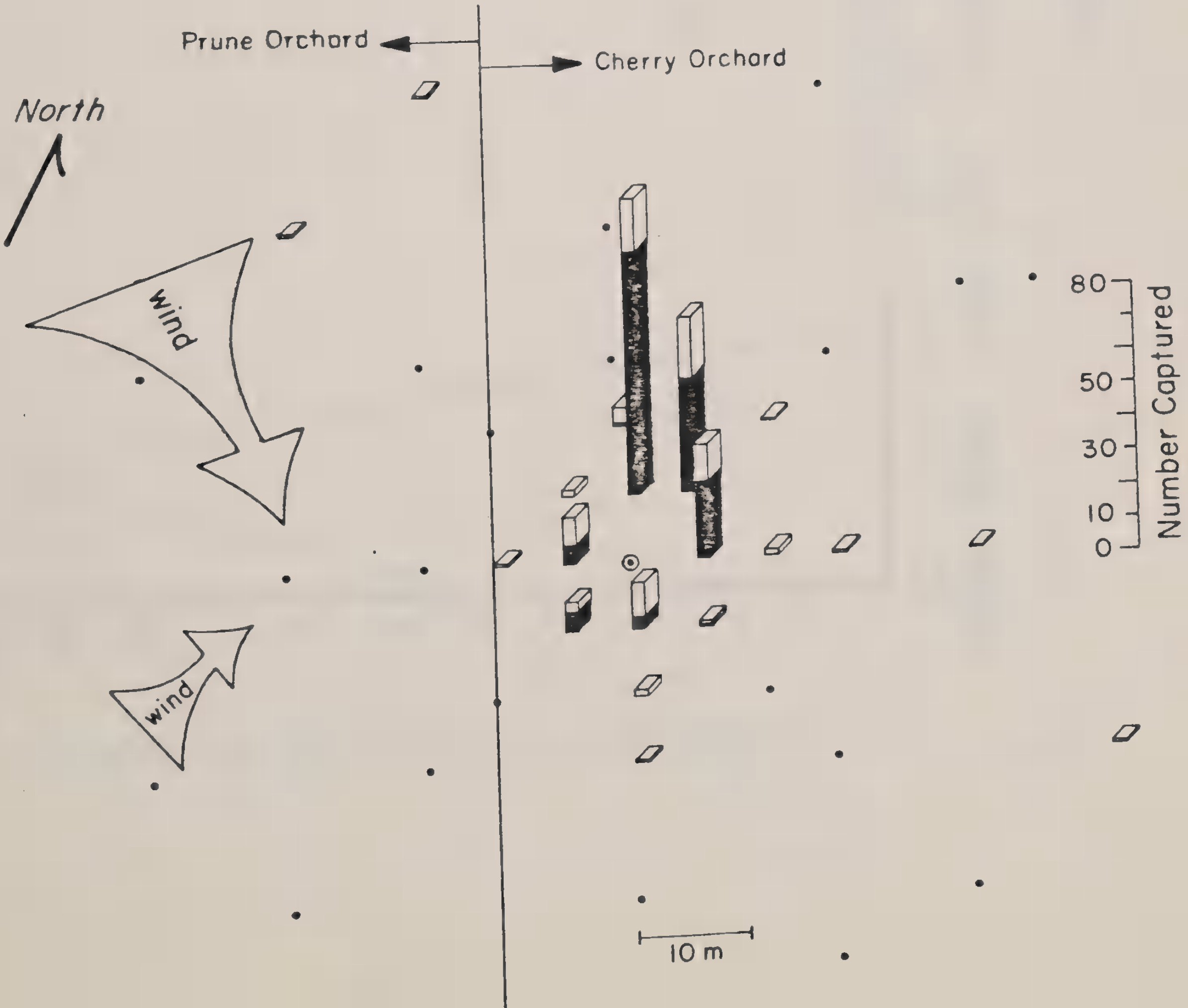

Prune Orchard
Cherry Orchard
North
wind
wind
80
50
30
10
0
Number Captured
10 m

Fig. 3

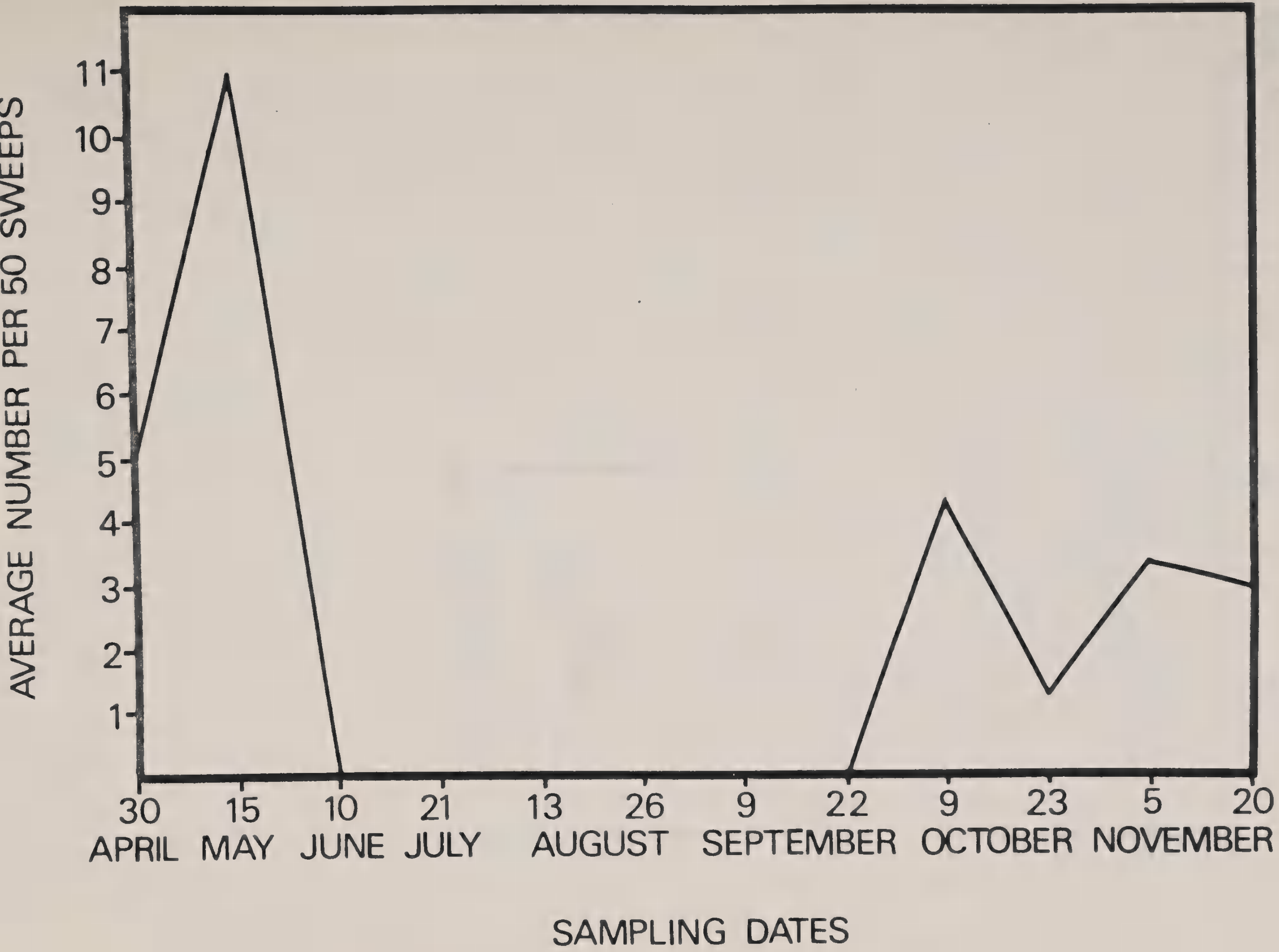
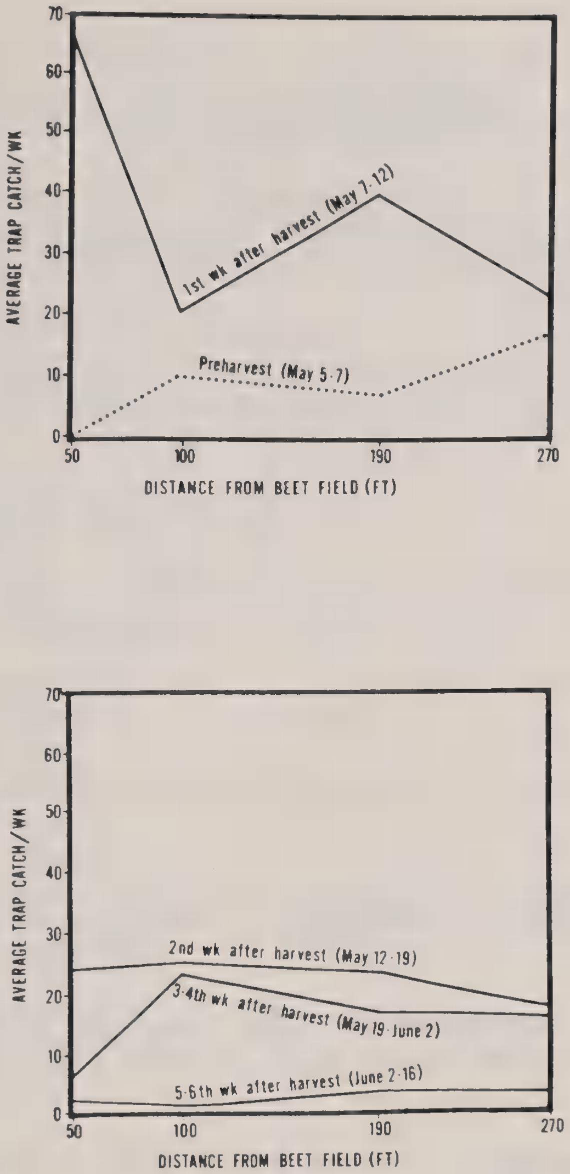

AVERAGE NUMBER PER 50 SWEEPS
11
10
9
8
7
6
5
4
3
2
1
30
APRIL
15
MAY
10
JUNE
21
JULY
13
AUGUST
26
9
SEPTEMBER
22
9
OCTOBER
23
5
NOVEMBER
20
SAMPLING DATES

Fig. 4


70
60
50
40
30
20
10
0
AVERAGE TRAP CATCH/WK
1st wk after harvest (May 7-12)
Preharvest (May 5-7)
50
100
190
270
DISTANCE FROM BEET FIELD (FT)
70
60
50
40
30
20
10
0
AVERAGE TRAP CATCH/WK
2nd wk after harvest (May 12-19)
3-4th wk after harvest (May 19-June 2)
5-6th wk after harvest (June 2-16)
50
100
190
270
DISTANCE FROM BEET FIELD (FT)

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SECTION II. Dr. J. M. Kloepper, Department of Plant Pathology, University of California, Berkeley, CA

This report covers work done by the Berkeley Plant Pathology group on X-disease in cherry from Oct. 1979 - March 31, 1981. By 1979 it was obvious that spiroplasmas were somehow associated with X-disease. In 1978, the consistent isolation of spiroplasmas from celery, lettuce and *Plantago* with X-disease was reported (7). Spiroplasmas were subsequently isolated from *Colladonus montanus* fed on X-diseased celery (5) while no isolates were obtained from leafhoppers fed on healthy celery. Spiroplasmas were also isolated by G. Nyland and R. C. Raju at U.C. Davis from X-diseased cherry in the field and were shown to be pathogenic to celery by A. H. Purcell.

Our initial hypothesis, therefore, was that spiroplasmas are the X-disease pathogen. We concentrated efforts during the winter of 1979-80 on improving isolation procedures for spiroplasmas in the greenhouse.

Media modifications

Existing spiroplasma media were compared for the isolation of spiroplasmas from infected plants. We used *Spiroplasma citri*-infected sweet orange and plantago for routine isolations. The isolation procedure used for the media modification studies is described in the following section. The percentage of successful spiroplasma isolates varied with different media, although all of the standard *S. citri* media supported growth of spiroplasmas after the primary isolations.

The following parameters of isolation media were tested:

1. osmolarity; 300, 500, 700, and 800 mOsm each with sorbitol and sucrose as the osmolarity regulators.

2. Serum; horse serum vs fetal bovine serum

3. pH; 7.2, 7.4, 7.6, 7.8 and 8.0

4. the presence of 0.006M Hepes buffer

5. the presence of 2% yeastolate

Each of the parameters was individually tested for possible improvement in percentage primary spiroplasma isolations compared to media SP4 (8) and ME-1 (6), which were the two best standard media for spiroplasma isolation. After extensive testing, we chose medium DG-2 as the best combination of components for isolations:

Autoclavable base	
PPLO broth base	20 g
Sorbitol	50 g
Tryptone	1 g
Peptone	1 g
Glucose	1 g
Fructose	1 g
Sucrose	1 g
Phenol red (0.2%)	10 ml
Distilled water	800 ml
(Difco agar)	(10 g)
Adjust to pH 7.4 with 1N NaOH	
Filter-sterilized additives	
CMRL 1066 10X without L-glutamine and $NaHCO_3$ (Gibco)	10 ml
Fresh yeast extract (100%)	10 ml
Yeastolate (2% solution)	100 ml
Horse serum (heated at 56° for 1 h) (Gibco GG-free)	170 ml
Penicillin (100,000 U/ml)	10 ml
Adjust to pH 7.4 with sterile 1 N NaOH	

Isolation Procedures

During early experiments on media modification, spiroplasmas were irregularly isolated from orange infected with S. citri, suggesting that populations of spiroplasmas in orange were generally low. In order to increase the sensitivity of isolation, two procedures were developed for the concentration of tissues. Young and medium-aged leaves were removed from the test plant and were surface-sterilized in 1.5% sodium hypochlorite for 2.5 min followed by three rinses in sterile water. Petiole and stem tissue (0.2 to 0.5 g) were homogenized in 10 ml phosphate-buffered sucrose (10% sucrose in 0.1 M phosphate buffer pH 7.4 and 618 mOsm) using a mortar and pestle. Samples were clarified by centrifugation at 500 x g (20°C) for 5 min and the supernatant was filtered through a 0.45 µm membrane filter using positive pressure and 0.1 ml aliquots were directly plated on DG-2 medium and maintained as undiluted liquid cultures (procedure 1).

In procedure 2, the clarifed filtered supernatant was recentrifuged in sterile screw-top centrifuge tubes at 25,000 x g at 17°C for 60 min. The pellet was resuspended in 0.6 ml DG-2 broth and 0.1 ml was inoculated onto agar plates and into 2.0 ml liquid DG-2. After drying by incubating at 28°C for 24 h, plates were placed inside closed plastic bags and incubated aerobically at 28°C for up to 5 weeks. Plates were examined at 2-day intervals for the presence of colonies or yellow color and liquid tubes were examined for color changes and for the presence of spiroplasma using the dark-field microscope.

Procedure 2 consistently yielded spiroplasma colonies on solid DG-2 medium without dilutions while some isolations were negative using procedure 1. Colony morphologies included lens-shaped without a dark center region and granular with satellite colonies. Isolation from large amounts of plant tissue (more than 0.12 g/ml) were frequently obtained only on solid medium and not in undiluted liquid medium; however, spiroplasmas were consistently isolated in undiluted liquid medium when less than 0.12 g host tissue/ml broth was used.

The effectiveness of DG-2 broth for isolations was compared to ME-1 and SP4 using isolation procedure 2, and DG-2 broth was superior to both ME-1 and SP-4 broth for the isolation of spiroplasmas without dilutions from the same plant tissues (Table 1). Spiroplasmas isolated in DG-2 broth were periodically not isolated in SP-4 or ME-1. The incubation time required for spiroplasmas isolated from plants infected with S. citri was the same using DG-2 and ME-1; however, the incubation time was less using DG-2, than ME-1 for isolation from plants with typical GVX, mild AY and stubborn-diseased citrus. DG-2, SP-4, and ME-1 agar plates were all useful for maintenance of isolated spiroplasmas.

Quantification of spiroplasmas in infected plants

The development of isolation procedure 2 allowed the quantification of spiroplasmas in plant tissue. In order to determine how many replications of one sample are necessary to calculate a reliable estimate of the spiroplasma population, the variability of the quantification procedure was measured. Five replications of leaf petioles from one stalk of celery infected with S. citri were sampled using procedure 2 described above, and the population of each sample was determined.

The population of spiroplasmas in petioles was compared with that in roots using celery infected with S. citri isolated from celery, orange with citrus stubborn disease, and corn with corn stunt disease. Four replicate samples from petioles and roots of each plant were used as described above to calculate the spiroplasma populations. The results (Table 3) show that the average spiroplasma population in petioles was approximately 1 log unit higher than the population in roots with each of the sample plants. No S. citri colonies developed on agar plates inoculated with homogenates of tissues from roots of orange with citrus stubborn disease. The minimum detectable number of spiroplasmas was 1.0×10^2 with the weight of tissues used in this particular experiment.

The distribution of spiroplasmas in foliage was determined by calculating the populations in new, medium-aged, and old celery and corn leaves. Leaf midveins were sampled from four corn plants with corn stunt disease and three celery infected with S. citri isolated from cherry.

Spiroplasmas were present in detectable numbers in leaves of all ages of corn with corn stunt and celery with S. citri (Table 4). However, populations decreased with increasing age of leaves with differences of 1 to 1.5 log units from young and old leaves.

The range of spiroplasma populations in different plant hosts was determined using celery, orange, and plantago infected with S. citri, corn with corn stunt disease, and S. citri-infected periwinkle which also had X-disease. The spiroplasma populations in foliage varied nearly 4 log units, from 5.0×10^1 cfu/g to a maximum of 3.1×10^6 cfu/g, among the different plant hosts sampled (Table 5).

Using the procedure reported here, the minimum detectable population is significantly below the minimum detectable level for ELISA (1).

Isolations from plants with X-disease in the greenhouse

The results of the quantification experiments indicated that our isolation procedure could detect low populations of spiroplasmas, even from plants with many inhibitors such as Vinca. We then used the same procedure to isolate sprioplasmas from plants with X-disease in the greenhouse. Source plants included celery, and Vinca with Green Valley X-disease, and celery and peach with peach yellow Leaf Roll X-disease. The percentages of spiroplasma isolations from these plants (Table 6) were compared with the frequency of isolation from celery infected via Colladonus montanu leafhoppers with a spiroplasma isolated from X-diseased cherry in the field.

The low frequency of spiroplasma isolations from plants with X-disease contrasts sharply with the consistent isolation of spiroplasmas from spiroplasma-infected celery. These results also disagree with the earlier report of consistent isolation of spiroplasmas from celery and lettuce with X-disease (7), and therefore these results were unexpected. The results suggest that spiroplasmas were not present in plants with X-disease from which no spiroplasmas were isolated. This would contradict the hypothesis that the spiroplasmas are the MLOs which cause X-disease. The quantification results demonstrated that even low populations of spiroplasmas could be isolated. Therefore, if the MLOs causing X-disease are spiroplasmas, spiroplasmas should have been consistently isolated from the X-diseased plants.

Two possible suggestions could account for this seeming discrepancy. First, the spiroplasma could be a morphological stage in the life cycle of the MLOs which cause X-disease. Second, the spiroplasmas may be distinct organisms which may or may not contribute to the X-disease syndrome. These possibilities are further discussed in the sections on field isolations, electron microscopy and identification of spiroplasmas.

Conclusions from Greenhouse work

The following conclusions may be drawn from the greenhouse work described in the preceding pages.

1. DG-2 medium is effective for isolation and maintenance of spiroplasmas.

2. An isolation procedure was developed which eliminated the need for "blind passages" (routine transfers of attempted isolates at 2 to 3 day intervals until growth occurs) and therefore allowed the processing of many more samples.

3. The isolation procedure allowed the quantification of spiroplasmas which indicated

    a) a variation of up to 4 log units in spiroplasma populations in different plant host species.

    b) spiroplasmas are present in higher populations in petioles than roots of the greenhouse plants

    c) populations decreased with increasing leaf age.

    d) extremely low populations could be detected including populations lower than those detectable by ELISA.

4. Spiroplasmas were consistently isolated from plants infected via leafhoppers with spiroplasmas originally isolated from cherry.

5. Spiroplasmas were isolated with extremely low frequency from plants with X-disease.

6. The ability to isolate *S. citri* from infected orange varied with the time of year suggesting a cycling of spiroplasma populations in orange.

1980 Field isolations

At the start of the 1980 field season, several spiroplasma strains had been previously isolated by the U.C. Davis plant pathology group and one strain by the U.C. Berkeley group under the direction of S. Thomson. These existing spiroplasmas were all part of bulk collections in which tissues from several trees were mixed into one sample for isolation, as is routine procedure for initial isolation attempts. Therefore, it was not possible to locate the precise tree with the spiroplasma infections. In order to detect individual trees with spiroplasmas, and in order to circument any possible population fluctuations as were observed in the greenhouse, we selected individual trees in 2 orchards for routine isolations. Fifteen mazzard and nine mahaleb cherry were chosen. The trees were known to be

diseased from 1979, and the designation of diseased was confirmed by symptoms at fruit maturity and by presence of MLOs as observed with electron microscopy. Tissue samples were collected weekly from March through September; leaves and flower or fruit peduncles were collected until after fruit fall when only leaves were collected. Isolations were atempted from the tissues as previously desribed using DG-2 broth medium.

In addition to the routine samples from the two sample orchards, bulk collections were made every two weeks from May through July from other orchards. Bulk collections consisted of petioles or fruit peduncle tissues from 4 trees together in one sample. Twenty-five bulk collections representing a total of 100 diseased trees were sampled at each collection date. One bulk collection (4 trees) of healthy trees was included at each sample. A total of 22 spiroplasma strains was obtained from the two routinely-sampled orchards (Table 7). All isolations were from diseased trees. The isolates were obtained during a 3-week-period from 4/30 - 5/21. After this time, spiroplasmas were not isolated from any trees, including those which previously yielded spiroplasmas. A total of 53% and 78% of trees in the two orchards yielded spiroplasma at some time of the year, which would tend to support the hypothesis that spiroplasmas may be the same as the MLOs which cause X-disease.

The total percentages of samples which yielded spiroplasmas were only 4 and 5% since spiroplasmas were only isolated during a 3-week-period. These data would tend to suggest that the spiroplasmas are not the disease-causing MLOs otherwise the total percentage of samples yielding spiroplasmas should be higher. If the spiroplasmas were simply a morphological form of the MLOs, the MLOs would grow into spiroplasmas during the incubation period of the isolation.

Even if the spiroplasmas are distinct organisms from the MLOs, they may have a pathogenic involvement along with the MLOs in producing X-disease. No spiroplasmas were isolated from healthy trees; however, most of the healthy trees were included in bulk samples which were sampled after the 3 wk period in which spiroplasmas were isolated. The question of a pathogenic role for the spiroplasmas can now be addressed by including healthy trees in a routine isolation procedure during 1981.

1981 Field isolations

The orchards sampled in 1980 were not available for use in 1981: one was destroyed and one was injected with tetracycline. Therefore, two different orchards were included in 1981, both with mahaleb root stocks. Ten to fiftenn diseased and healthy trees were selected for weekly samples. The initial diagnosis as healthy or diseased was confirmed by symptoms at fruit maturity and by electron microscopy. Weekly samples were conducted from April through May and processed as in 1980.

Spiroplasmas were isolated from both diseased and healthy limited trees in 1981. (Table 8) As in 1980, spiroplasmas were only isolated for a limited time preceding fruit maturity. The presence of spiroplasmas in 60% of healthy trees in one orchard suggests that the causal agent of X-disease is not a spiroplasma, and would tend to suggest that the spiroplasma does not contribute to pathogenicity, although the trees must be observe din subsequent years before concluding this.

Our data do not completely preclude the possibility that the spiroplasmas which were isolated from healthy trees are an early form of the MLO which causes X-disease, and that the healthy trees may develop X-disease symptoms in subsequent years as a result of the spiroplasma infection. We believe that this possibility is unlikely, however, since no MLOs were seen later in the season in the same healthy trees from which spiroplasmas were isolated in April. However, MLOs in diseased trees, which are often not detected with electron microscopy in April, develop into detectable populations by early May (Garrott, unpublished) and increase in concentration throughout the season.

Conclusions from field work - the following conclusions may be drawn from field work in 1980 and 1981

1. Spiroplasmas occur in a relatively large percentage of healthy and diseased cherry trees at some time during the year.

2. However, spiroplasmas are only isolated during a 2- to 3-wk-period prior to fruit maturity.

3. Cherry seems to be a poor host for spiroplasmas (since even healthy trees with spiroplasmas had undetectable populations during most of the year).

Electron Microscopy

Extensive electron microscopy has been conducted during this project either to aid in diagnosis or to study the in vivo morphology of MLOs. For diagnostic work, sections of petioles and leaf veins of greenhouse and field plants were processed as previously described (2) and examined in 70-nm-thick cross sections. MLOs were numerous in phloem sieve tube members of X-diseased celery and cherry (Fig. 1). MLOs were detected with electron microscopy in fruit peduncles on leaf petioles of X-diseased cherry beginning in mid April and populations increased throughout the season. There was a complete correlation between appearance of MLOs with electron microscopy and symptoms of X disease: no MLOs were detected in healthy trees or healthy greenhouse plants.

Most of our electron microscopic studies were designed to determine the in vivo morphology of the MLOs. Elucidation of in vivo morphology should help determine whether the spiroplasmas and the MLOs are the same or distinct organisms and should relate to results from the spiroplasma isolation experiments. Thicker sections of plant material were used in these studies (200 nm) in order to increase the depth perception, and both cross and longitudinal sections were used.

Plant materials examined included X-diseased cherry from which spiroplasmas were isolated, X-diseased cherry from which spiroplasmas were not isolated and celery infected with a spiroplasma which was isolated from X-diseased cherry. Evidence of helical morphology was detected only in those plants from which spiroplasmas were isolated. No helicity was detected using either cross or longitudinal thick sections of most X-diseased cherry. These results support the results from the isolation experiments and tend to suggest that the spiroplasmas are distinct organisms from the MLOs.

Recognizing the problems of interpretation of electron micrographs, we have recently been using high voltage electron microscopy (HVEM). HVEM allows the visualization of 1 μ thick sections which allow the discernment of an organism's true morphology without the interpretation required with thinner sections. For example, spiroplasmas were obviously present as helical organisms in phloem of celery from which spiroplasmas were isolated (Fig. 2). The in vivo morphology of MLOs in X-diseased cherry ranged from filaments (as previously reported by the U.C. Davis plant pathology group with X-diseased peach (3), to ovoid forms (Figs. 3 and 4), however, no helicity was detected in X-diseased cherry from which spiroplasmas were not isolated.

FIGURE LEGENDS:

Fig. 1. Cross sections (70 nm thick) of cherry with X-disease. Note presence of pleomorphic mycoplasma-like organisms (MLOs).

Fig. 2. High voltage electron microscopic (HVEM) examination of celery infected with spiroplasmas isolated from cherry with X-disease. Only helical MLOs were present. Spiroplasmas were cultured from the plant.

Fig. 3. and 4 1 µ thick HVEM section of X-diseased cherry peduncle. Note ovoid and filamentous MLOs present in several planes of view. Attempts to culture spiroplasmas from this tree were negative.

Identification of cherry spiroplasmas

Spiroplasma strains isolated from X-diseased and healthy cherry were individually identified using the spiroplasma deformation and growth inhibition tests. All strains were indistinguishable from *S. citri* using these tests.

Pathogenicity tests are currently in progress with the cherry spiroplasmas. Each strain is being injected into either *Colladonus montanus* or *Circulifer tenellus* leafhoppers which are being used to transmit the spiroplasmas to celery and cherry in greenhouse studies. The symptoms produced by the cherry spiroplasmas are being compared to symptoms of X-disease and to symptoms produced by *S. citri* isolated from stubborn-diseased orange.

Initial studies with 3 cherry spiroplasma strains indicate that the spiroplasmas do not cause typical X-disease. Celery plants exposed to leafhoppers injected with extracts of X-disease develop deformed young leaves, extreme chlorosis of older leaves, and root necrosis beginning 6 to 8 weeks after exposure to leafhoppers, and plants die within 2 months after first symptoms develop. In contrast, plants infected with cherry spiroplasmas do not deveop symptoms until 3 to 4 months after exposure to injected leafhoppers, and the plants survive 8 to 10 months. Symptoms of spiroplasma infection include leaf vein prominance, chlorotic leaf mottle and irregularly shaped leaf margins. Symptoms show some variation with the strain used; one strain produced extreme chlorosis and leaf roll.

We are currently concluding a study on the symptoms of *S. citri* in celery in conjunction with A. H. Purcell. The symptoms of the cherry spiroplasmas will be compared to those produced by *S. citri*. This work is still in progress; however it is already clear that the cherry spiroplasmas have both some similarities and some differences to symptoms of *S. citri*. It is possible that the cherry spiroplasmas may represent one or more distinct pathological groups (pathovars) of *S. citri*.

Summary - Our initial hypothesis that X-disease of cherry is caused by a spiroplasma is currently very much in doubt. It now appears that X-disease is caused by non-helical, non-cultivatable mycoplasma-like organism (MLOs) which are different organisms than the spiroplasmas. Spiroplasma infection of cherry apparently reflects a background infection of *Spiroplasma citri* in both healthy and diseased cherry. While cherry appears to be a poor host for spiroplasmas, the precise pathological role of the spiroplasmas is unclear. The isolation of spiroplasmas from healthy cherry in 1981 further suggests that they do not cause X-disease; however, it does not preclude their pathological involvement along with the X-disease MLOs in a pathogen complex. Future work will be aimed at clarifying the actual importance of spiroplasmas in pathogenicity using celery and cherry.

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Table 1. Isolation of plant spiroplasmas in various liquid media without subcultures.

Spiroplasma-infected Host	Medium	+ or -	Isolation in broth Average incubation time required[a] (weeks)
Stubborn-diseased	SP-4	-	-
orange	DG-2	+	4
	ME-1	+/-[b]	5.3
Plantago with	SP-4	+	4
S. citri	DG-2	+	2
Celery mild	SP-4	+	6
GVX[e]	DG-2	+	4.5
	ME-1	+	5

[a] Average time until spiroplasmas were detected using dark-field microscopy. Isolations were repeated six times.

[b] +/- indicates that spiroplasmas were sometimes not isolated.

[c] celery with typical symptoms of Green Valley X-disease

Table 2. Variation in spiroplasma population measurements from one sample[a]

Replicate	Population (cfu/g)[b]
1	1.0×10^6
2	1.0×10^6
3	2.6×10^5
4	2.0×10^5
5	8.0×10^5

[a] Five replicate petioles from one stalk of celery infected with *S. citri*.

[b] Colony-forming units per gram

Table 3. Spiroplasma population distribution in petioles compared to roots.

Plant sampled	Average population[a] (cfu/g)[b] Petioles	Roots
Celery with *S. citri*	7.8×10^5	5.3×10^4 *
Orange with citrus stubborn disease	3.0×10^3	1.0×10^2 *
Corn with corn stunt disease	1.7×10^3	3.6×10^2

[a] Average of four replications per treatment.

[b] Colony-forming units per gram.

*Indicates significantly lower mean compared to mean population in petioles (P - 0.05) using t-test.

Table 4. Spiroplasma population distribution in young, mid-aged and old leaves

	Spiroplasma population (cfu/g)[a]	
Leaf age	Corn with corn stunt disease[b]	Celery infected with *S. citri*[c]
Young	3.7×10^3 *	1.2×10^5 *
Medium	1.1×10^3 *	5.4×10^4
Old	1.5×10^2	3.4×10^3 *

[a] Colony-forming units per gram

[b] Average of four replicate plants.

[c] Average of three replicate plants.

*Indicates significant difference ($P = 0.05$) between means of populations from different leaf ages using t-test.

Table 5. The range of spiroplasma populations in greenhouse plants.

Infected plant	Spiroplasma population cfu/g[a] in sampled plant	
	Average[b]	Maximum
Celery with *S. citri*	6.0×10^5	2.6×10^6
Orange with *S. citri*	3.0×10^3	2.5×10^4
Corn with Corn stunt	2.7×10^3	2.5×10^4
Plantago with *S. citri*	1.1×10^6	3.1×10^6
Periwinkle with *S. citri* and X-disease	5.0×10^1	8.1×10^2

[a] colony forming units per gram

[b] Average of 3 replications per sample

Table 6. Frequency of isolation of various plant spiroplasmas

Disease/infection	Host plant	Isolation	No. of plants isolated from	Isolation frequency
Spiroplasma from X-diseased cherry	celery	+	9	consistent[a]
Green Valley X-disease	celery	+/-	12	2/34
	vinca	+/-	5	2/31
Peach Yellow leaf roll X-disease	celery	-	52	0/52
	peach	+/-	12	2/12

[a] consistent = over 85% successful isolations.

Table 7. Isolation of spiroplasmas from X-diseased cherry

Field 1980

Source tree	No. of sampled trees	No. of trees yielding spiroplasmas	Percentage spiroplasma infected trees	Dates of spiroplasma isolations	Total no.of samples during the season	Total no. positive isolations	Total percentage samples yielding spiroplasmas
Mazzard	15	8	53%	4/30,5/14	289	11	4%
Mahabeb	9	7	78%	5/14,5/21	229	11	5%

Table 8. Isolations of spiroplasma from healthy and diseased cherry, Field 1981.

Orchard No.	Diseased or healthy	Number of sampled trees	Number of trees yielding spiroplasmas	Percentage spiroplasma infected trees	Dates spiroplasma isolations
1	diseased	15	0	0	---
	healthy	10	0	0	---
2.	diseased	10	4	40%	4/1,4/14
	healthy	10	6	60%	4/1,4/14

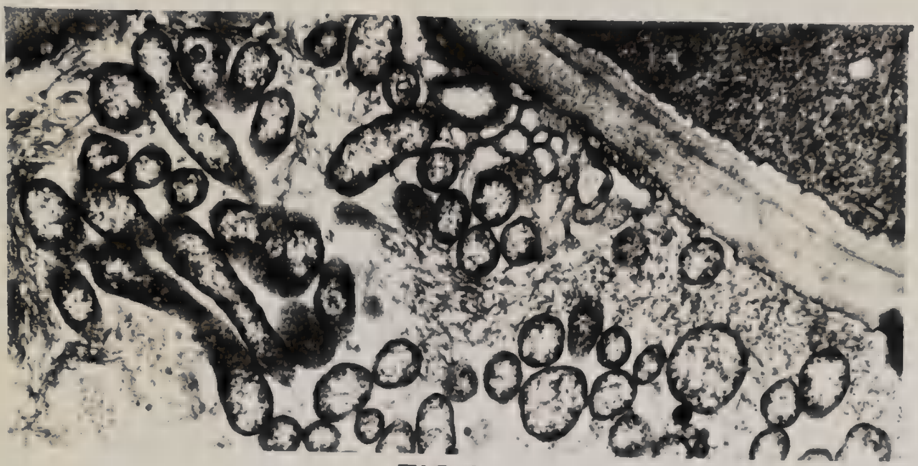

FIG.1

FIG.2

FIG.3

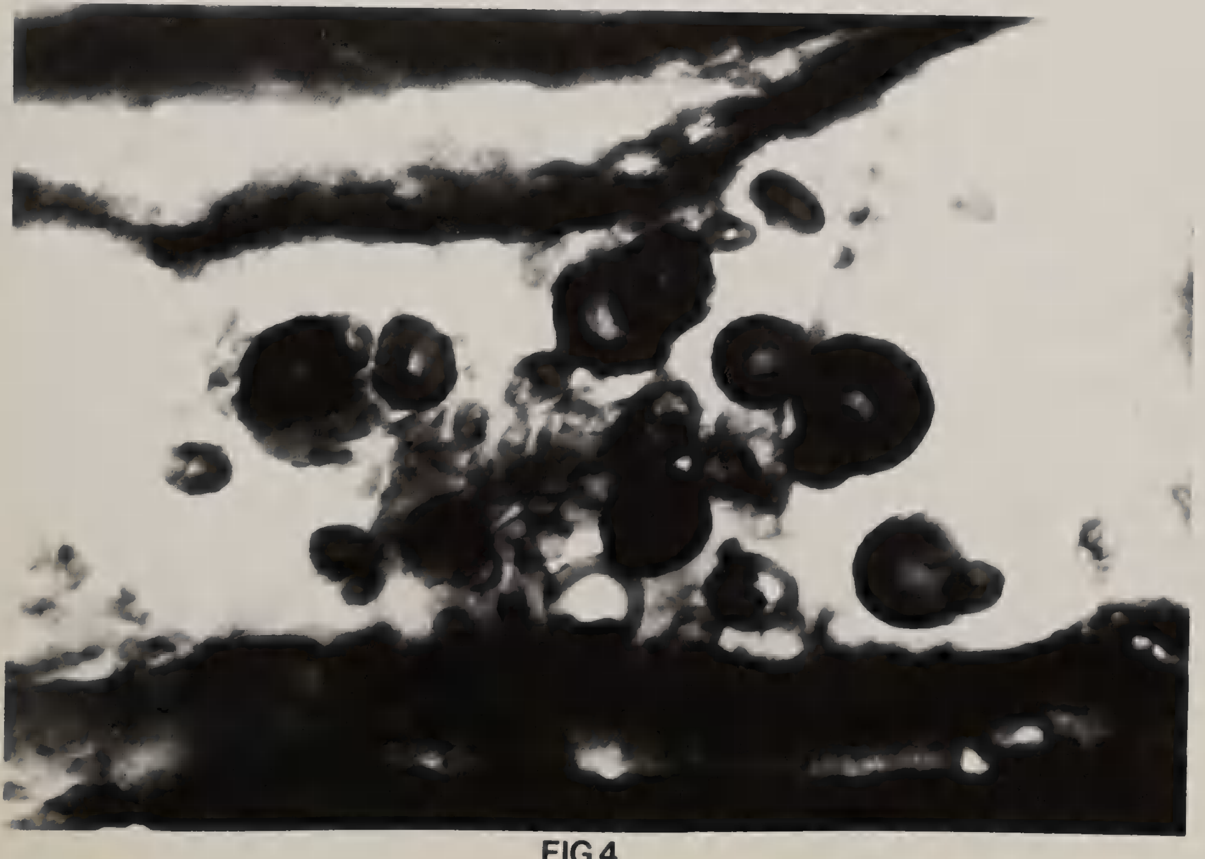

FIG.4

SECTION III.

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The principal objectives of our phase of the project were (1) to continue our attempts, in cooperation with Dr. A. H. Purcell, University of California, Berkeley, to prove pathogenicity of the Spiroplasma we isolated from buckskin trees; (2) improve methods and materials for chemotherapy of buckskin; (3) screen wild plants in and around orchards as possible reservoirs of infection of the buckskin pathogen; and (4) improve culture media, isolation, and serological techniques with the infectious agent that is associated in cherry with the buckskin disease. We shared objective #4 with Dr. Kloepper at Berkeley with our results not completely consistent with his.

Chemotherapy

Terramycin is efficacious in remitting symptoms of cherry buckskin in affected trees on Mazzard rootstocks. In case the infectious agent should become tolerant to Terramycin, a substitute chemical would be desirable. Two chemicals that had shown some promise were tested in affected trees in San Joaquin County in comparison with Terramycin.

Methods for comparing chemical

Terramycin source was pear decline tree formulation available commercially. Two other chemical CA-AFN and TC-2216 were experimental chemicals obtained from Dr. Thirumalachar of Walnut Creek, CA. Chemicals were injected with the Reil injector in 1 liter water for each tree. Checks were injected with water only. Injections were made March 6, 1979 and March 8, 1979 when the cherry bloom was in the "popcorn" stage.

Table 1. Injections of chemicals in cherry for Buckskin Control

Chemical	Date	Number of trees	Dosage (a.i.)	Disease Rating	
				Before injection	After injection
Terramycin	3/6/79	20	2g/tree	10[a]	8
CA-AFN	3/8/79	10	10g/tree	7	11
TC-2216		10	10g/tree	8	10

[a] Trees rated 0-16 from no symptoms to 100% diseased. Numbers represent averages of trees tested.

Terramycin reduced the disease but the other two chemcials had no beneficial effect.

Effect of time and number of treatment

Additional treatments were made in cherry trees on Mahaleb root affected with buckskin to determine the best time and number of annual treatments to determine if affected trees with primary symptoms of decline could be saved and brought back into production. The second objective was to determine if the disease could be prevented from spreading in the orchard by treating unaffected trees in orchards where epidemic increase was occurring. We were able to work on the first objective but had to defer work on the second.

In 1980, 327 diseased trees on Mahaleb rootstock and 175 trees on mazzard rootstock in two orchards were treated. The trees on mahaleb rootstock were showing mild to severe tree symptoms of disease and those on mazzard had shown fruit symptoms. Affected trees on Mahaleb usually die within 2 or 3 years after appearance of symptoms. Our tests were to determine if these trees could be saved and brought back into production. Some trees received a single injection beginning in June and a second and third injection in July and August. Others received single injections in July and August and some of these received additional injections at monthly intervals to December.

The results showed that trees receiving single monthly injections in June to December performed as well the following year as trees receiving multiple injections. As expected, affected trees on mazzard rootstock produced eseentially normal crops of fruit the year following injection treatments with Terramycin. We were able to show that injection of trees on mahaleb, if done in the early stages of symptoms before severe damage occurs at the graft union, is highly beneficial. The best time of treatment is when symptoms are first detected. Treatments made in October or later into the dormant season are most likely to be phytotoxic.

Isolation of spiroplasma from cherry trees with symptoms of cherry buckskin

Introduction and abstract

Earlier we had isolated spiroplasma from plants with symptoms of aster yellows, pear decline and peach X-disease. We were able to isolate spiroplasmas from cherry with symptoms of buckskin. We did not isolate any spiroplasma from surface-sterilized tissue of healthy plants. Our attempts at proving Koch's postulates for pathogenicity of our isolates is covered in detail in Part I of this report as cooperative work with A. H. Purcell.

Materials and Methods

Previous studies indicated that fruit pedicels were the best sources for spiroplasma isolation. Hence all the isolations during 1979-81 were carried out with fruits from diseased trees. At least one healthy tree per ten diseased trees was included as control. Isolations were attempted from three different orhcards. Fruit pedicels were removed and used in isolation studies. Isolations were done using ME-1 or ME-5 medium (3, 5) with the techniques described previously (3, 4). Spiroplasma isolates were characterized by the procedures described by Raju et al., (2, 3).

Antisera were produced (2) in New Zealand white rabbits against two cherry isolates. Serologic relationships of spiroplasmas from cherry with other spiroplasmas were studied by growth inhibition, organism deformation (2, 3), and ELISA (1, 3).

Known healthy and diseased cherry plant samples were tested by ELISA according to the methods described previously (1).

Results

Spiroplasmas were inconsistently (Table 1) isolated from diseased cherry. All the positive isolations were obtained before fruit maturation (April-May). Additional attempts to isolate spiroplasma from the same trees were not successful. We were not able to isolate spiroplasma from healthy trees (Table 1).

All cherry spiroplasma isolates produced typical fried-egg colonies on ME-1 or ME-5 agar medium. No cell wall was observed in electron-microscopy studies. Morphologically and ultrastructurally the isolates were indistinguishable from *Spiroplasma citri*. Serum was required for growth and the organism can utilize arginine. No growth was observed at 37°C and the optimum temperature for growth was 31°C. Serologically the spiroplasma isolates from cherry were identical to *S. citri*, but shared some common antigens with honeybee spiroplasma and corn stunt spiroplasma.

ELISA was not able to distinguish healthy and diseased cherry readily when leaf, stem, fruit pedicel, and root samples were used. The A_{405} nm values in ELISA with healthy and diseased materials were almost the same, even though occasionally weak color reaction was observed with diseased plant material when fruit pedicels were used. This is in contrast with excellent ELISA color reactions we obtained with corn stunt or brittle root of horseradish (1, 3). At present, ELISA is not reliable in distinguishing healthy cherry from diseased using the antisera prepared against spiroplasma isolated from cherry.

TABLE 1. Isolation of spiroplasmas from X-disease affected and healthy cherry.

Plant source	No of[a] trees sampled	No. of samples tested	No. positive[b] for isolation
A. Diseased			
1. Sweet cherry on Mahaleb rootstock	31	480	32
2. Sweet cherry on Mazzard rootstock	33	322	26
B. Healthy			
1. Mahaleb	3	84	0
2. Mazzard	3	78	0

[a] Trees were tested during 1979-81.

[b] Positives were from fruit samples.

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Epidemiology

Possible alternate hosts and reservoirs

One of our original objectives was to screen plants in and around cherry orchards to learn if some might be serving as reservoirs for the pathogen. Observations had indicated that, in epidemic situations, spread was from outside the orchards as indicated by disease gradients. We used the ELISA serological technique to screen weed and cultivated herbaceous plants for MLO infection. Our results to date are inconsistent and inconclusive. Five wild plants in and in the vicinity of cherry orchards with buckskin were positive with the ELISA test using antisera made against a spiroplasma isoalted from diseased cherry. These were Johnson grass, dandelion, curly dock, mustard, and wild lettuce. Plants that tested positive were maintained in the greenhouse and retested intermittently. The results were inconsistent in that plants testing positive on one or more occasions became negative and in no case were we able to isolate spiroplasma from any of the wild plants.

Conclusions and recommendations

Serious doubt has been cast on the hypothesis that spiroplasma are the causal agent of cherry buckskin. This is based on the failure to consistently isolate spiroplasma from diseased trees, failure to detect spiroplasma in situ, and the limited success of the pathogenicity tests using vectors injected with the Spiroplasma. Furthermore, Spiroplasma were isolated from healthy cherry trees as well as diseased trees. Further work is required to clarify some conflicting results and confirm others.

Chemotherapy with terramycin is a feasible and valuable control method for cherry buckskin in trees on Mazzard root. Trees on mahaleb root, if treated early enough after infection takes place, can be saved and returned to economic use. Since the damage to the tree is at the graft union in trees on mahaleb, complete systemic distribution of the chemical including the area of the graft union is required. Further work is necessary to improve treatment methods for trees on mahaleb.

Several weeds in and in the vicinity of cherry orchards were shown to be infected with MLO by the ELISA method. The role of weeds as reservoir hosts and in the disease epidemiology remains to be demonstrated.

The limited successful transmission of a disease agent present in axenic cultures of spiroplasma keeps open the possiblity that the buckskin agent is a spiroplasma. However, most of the celery plants exposed to injected leafhopper vectors showed disease symptoms different from those considered typical for buckskin disease in celery. Therefore the hypothesis of a mixed culture has not been disproved.

The marking and release of vectors showed that dusting with fluorescent powder is a reliable method of marking; that density decreases exponentially with distance from dispersal point; that yellow sticky board trap catches are difficult to convert to accurate population indices.

Population monitoring of vectors from sugar beet fields adjacent to orchards showed a marked increase in numbers of insects in the orchards immediately after beet harvest. The role of this migration in the incidence of disease was not verified since captured insects did not transmit disease to test plants in the greenhouse. Possible seasonal variation in infectivity remains to be studied. Sugarbeets remain a suspect host in the field since they were injected with the buckskin pathogen in greenhouse transmission tests with the beet leafhopper.

Greenhouse tests showed that young weed plants are very much more easily infected by infective leafhoppers than older plants. Young beets like young weeds may also be more easily infected.